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WHOLE-BODY PROTEIN METABOLISM IN MATURE AND GROWING HORSES RECEIVING PREDOMINANTLY FORAGE DIETS

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of
Science in the College of Agriculture, Food and
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By

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ABSTRACT OF THESIS

WHOLE-BODY PROTEIN METABOLISM IN MATURE AND GROWING HORSES RECEIVING PREDOMINANTLY FORAGE DIETS

There has been limited investigation as to whether a predominantly forage-based diet can provide adequate amounts of limiting amino acids (AA) to horses. The first objective was to determine if AA supplementation of AA believed to be limiting to protein synthesis in forage-based diets would affect measures of whole-body protein metabolism in sedentary mature horses. The effect of forage type (timothy or alfalfa) and AA supplementation (lysine, threonine or histidine) on plasma urea nitrogen (PUN) and AA concentrations and measures of whole-body phenylalanine kinetics were evaluated. There was no effect of either forage type or AA supplement on rates of whole-body protein synthesis ($P > 0.05$). The second objective was to determine the effects of either timothy or alfalfa hay supplemented with either a high or low protein ration balancer on measures of whole-body protein metabolism in yearling horses. The effect of forage type and the ration balancer protein level on concentrations of PUN, plasma AA and measures of whole-body phenylalanine kinetics were evaluated. There was no effect of treatment on average daily gain ($P = 0.18$). When horses consumed the alfalfa-based diets, rates of phenylalanine flux, oxidation and use for protein synthesis were greater than when they consumed timothy-based diets ($P < 0.05$). Phenylalanine use for protein synthesis was not affected by the protein level of the ration balancer ($P = 0.3$). Yearling horses achieve greater rates of protein synthesis when fed alfalfa-based diets, compared to timothy-based diets, supplemented with a low protein ration balancer.

Keywords: Horse, Amino acid supplementation, Ration balancer, Forage-based diets, Whole-body phenylalanine kinetics

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November 15, 2018

WHOLE-BODY PROTEIN METABOLISM IN MATURE AND GROWING HORSES RECEIVING PREDOMINANTLY FORAGE DIETS

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Chapter 1: Introduction

Horses evolved over millions of years as a grazing and foraging species that have adapted to subsist primarily on plant-based fiber sources. Formulating diets for horses can be challenging, partially due to the unique qualities of their gastrointestinal tract and the industry's expectations of horses' athletic ability. The National Council of Research (NRC) provides feeding guidelines for horses that are exercising, growing, and for those that have reached maturity, as well as mares in different stages of lactation and gestation (NRC, 2007). Estimations based on averages of previously performed research, which individually all have their own limitations, comprise the sourcing for feeding guidelines in the NRC.

Horses are a non-ruminant species with a glandular and a non-glandular portion of their stomach. It is known that in the distal glandular portion of the horse's stomach hydrochloric acid is continually secreted, but that saliva production only occurs during mastication (Alexander, 1965; Pagan, 1998). For a horse that is continuously grazing, saliva and feed intake help to buffer gastric acid secretions. However, for a horse with a perpetually empty stomach, gastric acid secretions can splash the non-glandular portion of the stomach during movement, deteriorating the epithelial lining. Consequences of this are serious medical concerns in horses, gastric distress and ulceration in the non-glandular portion of the stomach, which can in some cases lead to colic. Under certain circumstances, often when horses consume large grain meals, portions of starch that are undigested in the small intestine become rapidly fermented in the large intestine (Pagan et al., 2007). As a result, lactic acid production increases, and subsequently the pH of the hindgut decreases, which can lead to hindgut acidosis. Symptoms of hindgut acidosis

include irritation and/or damage to the intestinal mucosa and hindgut ulcers. Adequate forage intake and providing concentrate to horses thoughtfully help to mitigate and prevent these conditions.

Although there is no direct forage or fiber requirement for horses, it has been recommended that horses consume a minimum of 1% of BW daily as forage on an as fed basis (NRC, 1989), with levels higher than that being preferable in many cases. As reviewed by the NRC, the average voluntary dry matter intake of different feeds by mature horses were found to range from 1.8-3.2% of BW/d (Aiken et al., 1989; Crozier et al., 1997; Dulphy et al., 1997a; Dulphy et al., 1997b; Heusner, 1993; Marlow et al., 1983; NRC, 2007). In growing horses, the average voluntary dry matter intake of different feeds was found to range from 2-3% of BW/d (Aiken et al., 1989; Cymbaluk et al., 1989; Guay et al., 2002; LaCasha et al., 1999; McMeniman, 2000; NRC, 2007).

A symbiotic relationship exists between the horse and microbial populations in the hindgut during fiber digestion. As microbes digest cellulose and hemicellulose from fiber sources, which would otherwise not be able to be digested by the horse, volatile fatty acids (VFAs) are released as a byproduct of microbial fermentation. Therefore, adequate forage intake is important, not only for gastrointestinal health, but also as a source of energy for the horse. Research regarding the ability of forage to meet energy and protein requirements in horses has received relatively less attention than concentrates as energy and protein sources. When ponies consumed a typical equine diet, it was found that the production of VFAs in the cecum may be capable of meeting up to 30 percent of a sedentary pony's energetic requirements (Glinsky et al., 1976). More recently, research has shown that when fed a medium quality grassland hay, energy needs in sedentary

mature horses is capable being met and that hays of lower quality will meet more than 80% of energy needs (Vermorel et al., 1997a). A good quality forage alone can meet the protein needs of a sedentary mature horse, but as stated by Vermorel et al., horses consuming solely forage diets may experience energetic deficits. Despite protein supplementation, it has been reported that horses fed diets limiting in energy lost weight (Sticker et al., 1995). While this may be acutely beneficial for horses needing to lose weight, such as metabolic or overweight horses, chronic deficits in energy intake could have negative implications, particularly in growing horses.

The location and extent of digestion depends on the chemical composition and degree of processing of the ingested feed. Horses, unlike ruminants, are hindgut fermenters, and the majority of forages are digested post-cecally, in the large intestine (Gibbs et al., 1988). The majority of concentrate digestion in the horse occurs pre-cecally, before the cecum, in the small intestine (Gibbs et al., 1986). Enzymatic digestion occurs primarily pre-cecally, resulting in amino acids available for absorption, whereas microbial digestion occurs post-cecally, resulting in ammonia and microbial protein. The ability of the hindgut to absorb amino acids is still a topic that is being investigated. The hind gut digestibility of a feed does not necessarily equate to the absorbability of the feed, digested feeds must be absorbed in order to be used by the animal. There is evidence that hindgut cationic and neutral amino acid transporters are present in the equine large intestine in vitro (Woodward et al., 2010). In ponies, it has been demonstrated not only that free lysine can be transported across the brush border membrane in the colon in vitro, but that the colon also has a greater capacity and affinity for lysine transport than in the jejunum (Woodward et al., 2012). However, the extent to

which these transporters contribute free amino acids to different pools throughout the body is currently unknown (Hendriks et al., 2012). Because the hind gut is located distal to the organ sites most associated with protein digestion to amino acids, specifically the stomach, small intestine and pancreas, the capacity for free amino acid absorption does not necessarily equate to the ability of the hindgut to absorb microbial amino acids. Although the relative abundances of cationic and neutral amino acid transporters from the hindgut have been categorized in vitro, the capacity, affinity and location of these transporters throughout the lumen of the hindgut have yet to be established . Other studies have used isotopic infusion methods to detect stable or radioactively labeled isotope in the circulating indispensable amino acid pool successfully (Slade et al., 1971), whereas another group was not able to detect the isotope (Wysocki & Baker, 1975). It is possible that in instances where labeled isotopes are ingested orally that the labeled amino acids could be formed proximal to the large intestine by microbes. Data regarding the proportion of microbial amino acids formed in the foregut compared to the hindgut is lacking in horses. Future research should be directed towards identifying the equid's capacity to utilize protein originating from forages in the hindgut.

This thesis includes a review of the literature surrounding measures of protein and dietary amino acids adequacy, current equine protein feeding practices, and implications of overfeeding protein. Two studies were conducted as a part of this thesis research. The objective of the first study was to determine whether lysine, threonine or histidine supplementation to either a grass or legume forage-based diet would result in improvements in measures of whole-body protein synthesis in mature horse at maintenance. The objective of the second study was to determine whether whole-body

protein metabolism of growing horses would be altered by either forage type or the protein content of the ration balancer.

Chapter 2: Literature Review

Measurements of Protein Requirements in Horses

Crude Protein requirements

In horses, protein comprises the largest non-water component in the body. Crude protein is the standard measurement of dietary protein content of livestock feeds in the U.S., and is calculated by obtaining the nitrogen content of the feed and dividing it by the assumed nitrogen concentration of protein, which is often 16% (Jordan and Myers, 1972). Because crude protein is the standard measurement, recommendations for protein intake are expressed as crude protein. For an average mature horse at maintenance, crude protein requirements can be calculated using the following equation where BW is the body weight of the horse in kilograms (NRC, 2007):

$$\text{CP requirement} = \text{BW} \times 1.26 \text{ g CP/kg BW/d}$$

This equation estimates that a 500 kg mature, sedentary horse requires 630g of CP daily. If this horse consumes the average 2.5% of their BW in dry matter daily, the CP% of their daily ration should be at least 5.5% CP to meet requirements (NRC, 2007). This equation was derived using a linear regression from the means of 12 studies that measured nitrogen intake and retention resulting in 813 mg CP/kg BW/d needed for zero nitrogen retention (NRC, 2007). Then, when the same data was fitted to a broken-line model, it was estimated that the requirement be 0.202 g N/kg BW/d, resulting in a CP equivalent of 1.26 g/kg BW/d. Using a 95% confidence interval for the data, it was then determined that the requirement lies between 1.08 and 1.44 g CP/kg BW/d. These numbers, 1.08

g/kg BW/d, 1.26 g/kg BW/d and 1.44 g/kg BW/d, provide the three levels of maintenance, minimum, average and elevated, similar to those described for energy requirements (NRC, 2007).

For growing horses, the equation changes to account for the efficiency of dietary protein use for gain, E, which is 0.3 for a yearling horse. Differences in E are categorized in the NRC for horses at different stages of growth, but generally as the horse ages, E decreases. Young horses are assumed to be in an elevated maintenance state because they are growing, and this is why 1.44 g CP/kg BW/d is used as the estimated maintenance requirement, as opposed to the 1.26 g CP/kg BW/d for the average, sedentary mature horse. This equation assumes a 79% digestibility for dietary protein and accounts for changes in protein requirements as the horse grows by multiplying average daily gain (ADG) by 0.20 g N/kg BW/d, the estimated nitrogen requirement (NRC, 2007).

$$\text{CP requirement} = (\text{BW} \times 1.44 \text{ g CP/kg BW/d}) + \left(\frac{\text{ADG} \times 0.20}{\text{E}}\right) / 0.7$$

Using this equation estimates that a 12-month-old horse predicted to weigh 500 kg at maturity requires 846 g of CP daily. If this horse consumes the average 2.5% of their BW in dry matter daily, the CP% of their daily ration should be 10.5% CP to meet requirements (NRC, 2007). The equation for CP requirements for growing horses was estimated by modeling the level of CP intake resulting in maximal nitrogen retention and ADG in horses fed coast-cross hay, corn and soybean meal (de Almeida et al., 1998), soybean meal and alfalfa (Ott and Asquith, 1986), and soybean meal and Bermudagrass hay (Ott and Kivipelto, 2002), (NRC, 2007).

Dietary amino acids requirements

The goal when feeding horses protein is to provide adequate amounts of amino acids to maximize protein synthesis and reduce excess waste. This is challenging because individual amino acid requirements have not yet been determined in horses. As a result, requirements are provided and attempted to be fulfilled using the crude protein measurement. However, not all endogenous nitrogen in the feed exists as protein, but can also exist as creatine, purine and ammonium salts and not all protein can be digested. Therefore, crude protein does not accurately reflect absorbable nitrogen.

Meeting amino acid requirements enable tissue, hormone, transporter, enzyme and protein synthesis to occur in the body (NRC, 2007). Proteins are comprised of polypeptide chains, which are made up of sequences of amino acids. Individual amino acids are needed by the body in varying quantities to support metabolic functions and maintain homeostasis, particularly in individuals that are growing, in different disease states or with genetic abnormalities. Indispensable amino acids, by definition, cannot be made *de novo* in adequate quantities by mammalian enzymes and thus need to be provided by the diet. The body also requires dispensable amino acids, but these can be made *de novo*. Rather than having a requirement for CP, horses, as do most animals, require the indispensable amino acids and adequate amounts of amino nitrogen to make the dispensable amino acids for normal metabolic function. A lysine requirement has been established for horses by the NRC, and will be discussed in the next section (NRC, 2007).

Limiting amino acids and ideal protein

When a diet provides inadequate amounts of one or more of the indispensable amino acids, protein synthesis cannot proceed further than the rate which is supported by the indispensable amino acid provided the most below its requirement, effectively limiting protein synthesis. The amino acid that limits protein synthesis is called a limiting amino acid, and there can be multiple limiting amino acids. Lysine is known to be the first limiting amino acid in many species, including growing horses (Hintz et al., 1971b; Potter et al., 1975; Ott et al., 1981) and more recently has been confirmed in sedentary mature horses (Malesky et al., 2013).

Horses cannot use amino acids that are provided in excess of the rate of protein synthesis supported by the limiting amino acid and amino acids cannot be stored in the body as free amino acids to any large extent. Formulating diets that provide the minimum quantity of each indispensable amino acid in the correct ratios to one another, rather than using the feed's total nitrogen content, has been used previously in swine research and is known as the 'ideal protein' concept (Chung and Baker, 1992). The first step in achieving ideal protein is to identify the requirement of the most limiting amino acid.

Using means from seven studies measuring diet composition, intake and nitrogen retention, a linear regression was used to estimate the minimum lysine intake of 36 mg/kg BW/d for sedentary mature horses (NRC, 2007). Using the same data, the optimum requirement for lysine was determined by identifying the

plateau achieved when nitrogen retention was equal to zero using a broken-line analysis (NRC, 2007). At a lysine intake of 54 mg/kg BW/d, the plateau in N retention for maintenance horses was achieved (NRC, 2007). For a 500 kg horse, this equates to a minimum requirement of 18 g lysine/d, while the optimum requirement is 27 g lysine/day. Being that the 500 kg horse requires 630 g of crude protein each day, 27 g/d of lysine is equivalent to 4.3% of the total daily crude protein requirement. Therefore, the horse's optimum requirement for lysine is calculated by the following equation, where CP is crude protein:

Lysine requirement = CP requirement × 4.3% of the CP requirement

A previous study tested four levels of lysine intake in mature horses and used plasma lysine concentrations to evaluate requirement (Ohta et al., 2007). Using a broken-line analysis, a plateau was reached at 0.47% of diet, which is equivalent to 72 mg/kg BW/d of lysine (Ohta et al., 2007). This estimate is greater than the NRC's recommendation for sedentary mature horses' daily lysine intake of 54 mg/kg BW/d (NRC, 2007). Limitations of this study include a small sample size and that no other methods, such as nitrogen balance, or plasma urea nitrogen analysis, were used in conjunction with their sample analysis. It is also possible that the NRC's daily lysine recommendation underestimates the requirement in sedentary mature horses, however more research is needed to confirm or deny this.

Similar approaches using averages from studies reporting ADG, diet, body weight and feed intake were used to estimate the lysine requirements in growing horses. A broken-line analysis was used to establish the lysine requirement for

horses aged 4 to 10 months, which is 168 mg/kg BW/d (NRC, 2007). For a horse aged 4 months weighing 168 kg, 669 g of CP are required daily, and 168 mg/kg BW/d of lysine equates to 29 g of lysine/d, which is 4.3% of this horse's CP requirement. This is the same requirement as the sedentary mature horse, and thus the equation for the lysine requirement of growing horses is the same. More research is required to determine if this value is correct for horses aged 11-18 months, however, studies have been conducted investigating lysine supplementation in growing horses that suggest other levels of lysine intake may be more suitable.

Another study used 12 weanling horses and six levels of lysine contents ranging from 0.25 to 0.70% of the total diet (Breuer and Golden, 1971). Weanlings were fed either one of two different forages, dehydrated alfalfa meal or ground bermudagrass, and a concentrate with varying levels of corn and soybean meal to create the six levels of lysine in two, 3x3 Latin squares (Breuer and Golden, 1971). It was determined from this study that average daily gain increased significantly when comparing gains from horses consuming the lowest level of lysine, to horses consuming 0.6% and greater of their total daily intake as lysine (Breuer and Golden, 1971). When horses consumed 0.6% of their total daily intake as lysine, this equated to 29 g lysine/day, which is what the NRC recommends for horses aged 4-10 months (Breuer and Golden, 1971; NRC, 2007). There are many limitations of this research, such as not providing the average age of the horses used and not knowing the chemical components of the

diets these horses received, but the data does support that the NRC's findings for weanlings and younger horses appear valid.

Another study provided 46 yearling horses with between 40-59 g lysine/d (107-158 mg/kg BW/d; average BW of 372.8 kg), in two experiments for 196 and 140 days, respectively (Ott et al., 1981). By varying the amount of soybean meal (SBM) in the concentrate (11%, 9%, or 3% SBM), comparing SBM to brewers dried grains (BDG) (11% SBM to 18% BDG) and adding lysine supplements to certain SBM concentrates, six different levels of crude protein and lysine were obtained in the concentrate portions of the yearling's diets. The percent of lysine with respect to the amount of total daily crude protein intake in the diets of the two experiments spanned from 3.6-4.8%, which is well above and below the NRC's recommendation of 4.3% (NRC, 2007). All horses were offered 1% of their body weight each day of Costal bermudagrass, and average hay intakes were 0.94% and 0.92% for experiment one and two respectively and all diets were isoenergetic (NRC, 2007; Ott et al., 1981). It was determined from experiment one that lysine intakes of 40 g/d (107 mg/kg BW/d) resulted in slightly slower growths, based on differences in girth gain (Ott et al., 1981). It is important to note that in experiment one, when horses consumed 48 g/d and 52 g/d (129-139 mg/kg BW/d) of lysine, there were no differences seen in girth gain and that there were no differences between all three dietary treatments with respect to any other growth parameter studied (Ott et al., 1981).

In experiment two, despite no differences in total intake, horses receiving the 12% CP soybean meal supplemented with lysine had the lowest feed to gain

ratio (Ott et al., 1981). Yearlings consuming the 14% CP soybean meal had the same gain per day ratio as yearlings consuming the 12% CP soybean meal supplemented with lysine (Ott et al., 1981). This implies that the same rates of growth, at a lower feed to gain ratio, were able to be obtained when horses consumed the reduced crude protein concentrate supplemented with lysine. From these findings, the authors suggest a minimum intake of 48 g lysine/day (129 mg/kg BW/d) for yearling horses (Ott et al., 1981). Another important conclusion of this study was that a ratio of 1.9 g lysine/Mcal digestible energy resulted in improved growth (Ott et al., 1981). From these results, it is difficult to conclude if the rate of lysine supplementation alone was the determinant for improved growth, or if it was the ratio of lysine to digestible energy that allowed for improved growth. Although improvements in growth or weight gain can indicate dietary protein adequacy, this is not a reasonable measure for protein requirements.

Using the indicator oxidation amino acid method, a study using six yearling horses measured the responses of whole-body protein synthesis in yearling horses receiving graded levels of lysine (Mastellar et al., 2016b). The experimental diets were formulated to be isonitrogenous and isocaloric (Mastellar et al., 2016b). Two concentrates were used in this study, with one containing free lysine, and the other containing an isonitrogenous amount of glycine and oats (Mastellar et al., 2016b). Concentrate was fed at 1.11% of BW/d and the two types of concentrates were mixed in different ratios to create six diets with lysine intakes ranging from 76 to 136 mg/kg BW/day (Mastellar et al., 2016b). All

horses received timothy hay cubes (7% CP) at 1.37% of BW/d. Despite half of the levels of lysine being provided above and the other half below the NRC's recommendation of 113 mg/kg BW/d, no differences in phenylalanine kinetics were observed between lysine intakes (Mastellar et al., 2016b). When a broken-line analysis of lysine intake and phenylalanine oxidation was performed, a plateau was unable to be identified (Mastellar et al., 2016b). This indicates that the diets consumed by yearlings in this study may have been deficient in another limiting amino acid other than lysine (Mastellar et al., 2016b).

Formulating diets to contain this 'ideal protein' is challenging because while a few studies focused on determining lysine requirements have been conducted, particularly in growing horses, studies targeting secondary or tertiary limiting amino acids in horses have provided inconclusive results. Studies have suggested that threonine could potentially be the second most limiting amino acid in both growing and mature horses; however more research is necessary to reach this conclusion (Graham et al., 1994; Staniar et al., 2001; Malesky et al., 2013; Mastellar et al., 2016a). Recently, a study was conducted in growing horses, evaluating threonine requirements using isotopic methods and blood metabolites (Smith, 2016). It was found that threonine requirements appear to be adequately met by typical of diets that meet the DE and CP requirements of yearling horses (Smith, 2016). Using six sedentary mature mares fed six levels of threonine ranging from 41 to 89 mg/kg BW/day; it was found that the threonine requirement in mature horses may be lower than recommended by the NRC (Mok et al., 2018, NRC, 2007). Using the ratio of lysine to threonine found in equine

muscle tissue, 100:61, a 600 kg horse requiring 54 mg/kg BW/day of lysine, results in a threonine requirement of 33 mg/kg BW/d for a 600 kg mature, sedentary horse (Bryden, 1991; NRC, 2007). Limitations of this study are that a narrow range of threonine intakes were used and the lowest level of threonine supplied just above recommended daily intake values. It was reported that dietary lysine intake was 2 mg/kg BW/d higher and histidine intake was >1 mg/kg BW/d higher than the estimated recommendations in all treatments (Lorenzo & Pateiro, 2013; Mok et al., 2018; NRC, 2007). Therefore, it is possible that another limiting amino acid, such as lysine or histidine, was more limiting than threonine in the experimental diets.

Recently, methionine has been investigated as another possible limiting amino acid; however, no effect of methionine level on growth or nitrogen balance was observed (Winsco et al., 2011). Histidine has also been suggested as another limiting amino acid in diets common to horses (Graham-Thiers and Bowen, 2011; Tanner et al., 2014), but histidine supplementation has not been previously studied in horses of any age. Because limiting amino acids are dependent on both the age of the horse and the specific ingredient composition of the diet, there is a need to investigate potentially limiting amino acids in horses of different ages, consuming different diets. Additional research is also needed to estimate the requirements of the other indispensable amino acids so that potentially limiting amino acids could be more readily identified.

Methods of determining dietary amino acid adequacy

Criteria for an "ideal" amino acid requirement study

When designing experiments to determine any nutrient requirement, multiple factors must be considered. Using multiple doses and a range of intakes of a test nutrient, such as a specific amino acid, is required to accurately detect a requirement. A minimum of four, but ideally six or more, levels of a test nutrient should be tested both above and below the estimated requirement (Baker, 1986; Pencharz and Ball, 2003). It is important to consider levels of toxicity and deficiency when selecting ranges of the test nutrient for experimental parameters. Dietary treatments also need to be isoenergetic and isonitrogenous to remove variability and achieve a closer estimate of the requirement of the test nutrient. To achieve isonitrogenous diets, the nitrogen source added to the dietary treatments should not share metabolic pathways with the test amino acid. For example, glycine is commonly supplemented to dietary treatments to make them isonitrogenous when lysine has been investigated as a test amino acid in humans because the metabolism of lysine and glycine are unrelated (Brewer et al., 1978; Rice et al., 1970). Another example of choosing a diet-balancing amino acid for dietary treatments occurs when studying phenylalanine as a test amino acid. In this scenario, tyrosine should not be used to balance dietary treatments because the first step of phenylalanine oxidation converts phenylalanine to tyrosine. Additional considerations with regards to experiments designed to establish dietary requirements for essential nutrients are reviewed by Baker (1986).

Tissue amino acid composition

The amino acid composition of muscle tissue is assumed to be representative of whole-body protein composition in growing horses (Bryden, 1991) and has been suggested as a proxy for estimating indispensable amino acid requirements (NRC, 2007). Using the ratio of lysine to the amino acid of interest in the muscle and the lysine requirement of the horse, which changes depending on factors such as age, exercise, stage of gestation or lactation, requirements for other indispensable amino acids can be estimated (Bryden, 1991; NRC, 2007). Although this method provides an approximate estimation of requirements, it has limitations. First, splanchnic extraction, the removal of dietary nutrients, such as amino acids, by the visceral organs and tissues (i.e. liver, pancreas, gastrointestinal cells) prior to entry into general circulation for homeostatic metabolism and maintenance, is not accounted for (Jourdan et al., 2011). Secondly, this method assumes that the amino acid composition of muscle tissue is representative of the amino acids needed by the animal for tissue accretion and repair. This allows muscle tissue to be used as a proxy for whole body protein composition. However, differences between amino acid profiles of muscle tissue and other body tissues such as liver or skin, have been seen in growing lambs (MacRae et al., 1993). Muscle tissues comprises 44-53% of the live weight of horses (Gunn, 1987), but the measure of protein from muscle does not account for other body proteins, for example protein transporters or proteins involved in immune function. As humans age, the ratio of muscle to fat decreases, and the same can be assumed for horses (Janssen et al., 2000). Unlike growing animals,

mature animals are not actively accreting muscle, and so maintenance requirements of these animals may not closely reflect the muscle profile. Therefore, obtaining a muscle tissue sample for animals of one age may not provide an adequate basis to estimate amino acid requirements for animals of another age.

The amino acid profile of equine milk has also been investigated as a proxy for whole body indispensable amino acid requirements of the foal empirically (Stamper et al., 2005; NRC, 2007). Other research confirmed, with the exception of histidine, leucine and valine, that the amino acid profiles of milk and muscle tissue are indeed comparable (Davis et al., 1994; Doreau et al., 1990; Wickens et al., 2002; Stamper et al., 2005).

It is important to note, however, that free and protein bound amino acid pools from different organs and tissues differ. For example, in swine it has been reported that samples from the intestinal mucosa, liver, and kidneys have different free amino acid concentrations (Bertolo et al., 2000). The same was seen in rats when free amino acids concentrations of samples from plasma, liver, brain, muscle and spleen were compared (Schurr et al., 1949). In cats, when comparing different proteins and the free amino acid composition of the liver, brain pancreas, muscle, bladder, kidney, urine and plasma, differences in concentrations were also seen (Tallan et al., 1954). It has been characterized in other species that muscle protein has a different amino acid profile than other tissues. However, because such a large portion of the body's total mass is muscle, and the major

non-water component of muscle is protein, feeding amino acids in a similar ratio to that of muscle tissue should in theory support adequate protein synthesis

Nitrogen Balance

Classically, nitrogen balance studies have been used to determine dietary protein adequacy in horses consuming different diets (Graham-Thiers and Bowen, 2011), during different stages of development (Hintz et al., 1971b) and during exercise (Freeman et al., 1988; Graham-Thiers and Bowen, 2011). There are different calculations that can be used to measure different nitrogen balance parameters, such as how much nitrogen is absorbed or retained. These are commonly expressed as percentages based on the amount of nitrogen absorption or intake.

Nitrogen absorption can be calculated be using the following equation and for the following equations, where N is always nitrogen (NRC, 2007):

$$\text{N Absorbed} = \text{N Intake} - \text{N excreted in feces}$$

Nitrogen retention, also called digestible nitrogen, can be calculated using the following equation (NRC, 2007):

$$\text{N Retention} = \text{N Intake} - (\text{N excreted in urine} + \text{N excreted in feces})$$

Nitrogen absorbed as a percentage of intake can be calculated using the following equation (NRC, 2007):

$$\text{N absorbed as a percentage of intake} = (\text{N Absorbed/N Intake}) \times 100$$

Nitrogen retained as a percentage of intake can be calculated using the following equation (NRC, 2007):

$$\text{N retained as a percentage of intake} = (\text{N Retained/N Intake}) \times 100$$

Nitrogen retained as a percentage of nitrogen that is absorbed can be calculated using the following equation (NRC, 2007):

$$\text{N retained as a percentage of absorbed} = (\text{N Retained/N Absorbed}) \times 100$$

The greater the percentage of the nitrogen intake that is retained, the lower the proportion of dietary nitrogen that is lost to the environment in the form of nitrogen losses from urine or feces. As the intake of a limiting amino acid increases, without exceeding the level of requirement, protein synthesis increases. Therefore, amino acid oxidation and excretion decreases, and urea synthesis and excretion also decrease. Assuming that treatments are isonitrogenous, when the level of amino acid intake exceeds the requirement, or exceeds the limiting status of another amino acid, the rate of protein synthesis becomes maximized. Therefore, the percent of dietary nitrogen retained, as well as the amount of nitrogen excreted in the urine, stabilizes. Generally, when amino acids are fed in excess of their requirements, a plateau in N retention is observed.

When used in conjunction with other methods to study dietary amino acid adequacy, nitrogen balance provides useful support; however, there are limitations to using nitrogen balance studies as the sole source of information. Data resulting from more recent studies suggests that nitrogen balance alone may not be a sensitive enough measure to determine specific amino acid requirements in horses, but it is capable of comparing nitrogen retention at different levels of crude protein intake (Antilley et al., 2007; Malesky et al., 2013). Using six yearling Quarter Horse fillies in a 3 x 3 Latin square design, nitrogen retention was evaluated as a response criterion for amino acid studies (Antilley et al.,

2007). Yearlings were fed a control diet with adequate amino acid provisions, a diet deficient in amino acids, but that was supplemented with urea and a diet deficient in amino acids, but that was supplemented with oral dosing of essential amino acids (Antilley et al., 2007). Significant differences were observed between amino acid sufficient and deficient diets with respect to nitrogen retention as a percent of nitrogen absorbed (Antilley et al., 2007). However, no significant differences were observed between amino acid sufficient and deficient diets with respect to nitrogen retention alone (Antilley et al., 2007). Therefore, it was concluded that nitrogen retention alone did not seem to be sensitive enough to distinguish between diets sufficient or deficient in amino acids (Antilley et al., 2007). Using six mature geldings in a 3 x 3 Latin square design, nitrogen retention was evaluated as a response criterion for determining the effects of three levels dietary lysine intake (Malesky et al., 2013). Similar to Antilley et al., no differences in nitrogen retention were seen among the three levels of dietary lysine intake (Malesky et al., 2013). For this reason, it is important for nitrogen balance studies to be used in conjunction with other methods to study dietary amino acid adequacy.

Another limitation of this method is that distinguishing between catabolized dietary amino acids and catabolized body amino acids in the urine is not possible, thus excretion of nitrogen balance alone does not provide the origin of nitrogen that has been excreted. The same is true for fecal nitrogen losses, where differences between excretion of endogenous and unabsorbed nitrogen in the feces are unable to be characterized. Nitrogen losses from uncollected

excretions such as hair, sweat, or saliva contribute towards the margin of error in nitrogen balance studies, often overestimating the nitrogen retained (Kopple, 1987; Antilley et al., 2007; NRC, 2007).

Concentration of plasma free amino acids

The rationale behind using concentrations of plasma amino acids as a response factor is that as dietary amino acids are consumed in excess of requirements, concentrations of those amino acids elevate in the blood. Increased concentrations of plasma amino acids indicate that the intake of an amino acid exceeded its biological requirement and becomes marked for catabolism and eventual excretion. Changes in dietary protein content and amino acid profile on plasma amino acid concentrations were initially investigated in dogs (Hier, 1947; Denton et al.,1953). The NRC has established that sedentary mature horses require 54 mg/kg BW/d of lysine, but a study testing four levels of lysine intake in mature thoroughbred horses resulted in a different conclusion (NRC, 2007; Ohta et al., 2007). Using plasma lysine concentrations, a break point in plasma lysine was obtained when horses consumed 0.47% of the experimental treatment as lysine, which provided 72 mg/kg BW/d, well over the NRC's current recommendation (Ohta et al., 2007). It is possible that the NRC underestimates the lysine requirements for sedentary mature horses; however, few references available in the literature for comparison currently exist. The same group used four mature horses in a 4x4 Latin square arrangement to estimate dietary threonine requirements using plasma amino acid concentrations as the response criterion (Yoshida and Ohta, 2017). Using plasma threonine concentrations in

response to four levels of threonine intake ranging from 0.37-0.5% of total daily intake, a break-point analysis was conducted and a plateau was achieved at 0.41% (Yoshida and Ohta, 2017). The NRC recommends, based on the ratio of horse muscle tissue lysine to threonine concentration, that threonine be fed at 61% of the lysine requirement (NRC, 2007). From this study's research, a recommendation for the threonine requirement of sedentary mature horses was estimated to be 67-80% of the lysine requirement, well above the NRC's recommendation (Yoshida and Ohta, 2017). This study demonstrated that plasma amino acid concentrations of threonine were responsive to dietary threonine supplementation; however, more research is needed to discern if this threonine requirement for sedentary mature horses is indeed valid.

Recently, a study in growing horses showed that as the amount of lysine consumed by the horses increased, plasma lysine concentrations also increased in a linear manner (Mastellar et al., 2016b). Plasma amino acid concentrations of mature horses at maintenance have also proven to be sensitive to changes in dietary amino acid concentration (Wagner et al., 2013). Plasma threonine concentrations from sedentary mature horses at maintenance have been shown to respond to dietary treatments when fed six graded levels of threonine intake (Mok et al., 2018). Plasma threonine concentrations of growing horses were found to be different when horses were fed a basal diet, and a diet supplemented with threonine (Mastellar et al., 2016a). Because amino acid pools respond quickly to dietary changes, adaptation times to certain diets are able to be shortened (Watanabe et al., 1998). Responses of plasma amino acid pools to dietary changes

in multiple species such as rats, pigs and horses are rapid, and typically occur within one to two days (Watanabe et al., 1998; Moehn et al., 2004; Ohta et al., 2007).

When used in conjunction with other methods used to determine amino acid requirements of the horse, plasma amino acid concentrations can provide accurate estimates, however when used alone there are limitations. The same assumption used for muscle tissue or milk to be used as a representative of whole body indispensable amino acids is required in order to use plasma as a proxy for whole body protein composition. However, amino acids are not capable of passive diffusion and thus must be transported. The same limitation, that amino acid pools within different organs and tissues differ, also applies to using plasma as a proxy for whole body amino acid composition (Bertolo et al., 2000).

Concentrations of plasma urea nitrogen

Plasma urea nitrogen concentrations have been used as an indicator of amino acid catabolism in many species. There are two primary fates of dietary amino acids after they are absorbed from the intestine: catabolism or protein synthesis. When amino acids are catabolized, the amino moiety enters the urea cycle in the liver and is ultimately converted to urea destined for excretion as urine and the carbon backbones are converted to CO_2 and are exhaled. By comparing either pre-feeding or post-feeding plasma urea nitrogen concentrations between treatments, dietary amino acid catabolism differences can be identified. This method has been used as a measure of whole-body amino acid catabolism in humans, ruminants, horses and swine (Preston et al., 1965; Coma et al., 1995;

Layman et al., 2002; Graham-Thiers and Kronfeld, 2005; Kohn et al., 2005; Winsco et al., 2011; Mantovani et al., 2014). In swine, this method has been used by itself to successfully determine lysine requirements (Cameron et al., 2003; Knowles et al., 1997; Sparks, 1998). As the intake of a limiting amino acid increases, levels of plasma urea nitrogen decrease until the limiting amino acid requirement is fulfilled. Once the requirement for the limiting amino acid requirement has been met, plasma urea nitrogen concentrations plateau. Plasma urea nitrogen concentrations decrease as the level of supplementation of limiting amino acids increase because other dietary amino acids are able to be used for protein synthesis and a smaller proportion of those amino acids are catabolized to urea.

A limitation of this method is that regardless of whether the source of the amino acid destined for catabolism is endogenous or exogenous, urea formation is possible, so distinguishing its origin is not possible. Another limitation of this method is that ammonia is absorbed from the hindgut, so plasma urea nitrogen concentrations reflect not only the catabolism of amino acids, but the absorption of nitrogen from the hindgut as well (Bochroder et al., 1994). Additionally, because it can take 24-48 hours for forages to pass through the equine gastrointestinal tract, getting a true fasted urea nitrogen estimate is difficult. During fasting, endogenous breakdown of body proteins could affect circulating urea nitrogen concentrations as well. For all of these reasons, plasma urea nitrogen concentrations are inadequate as a rapid response criterion, but may be

best used in conjunction with other methods used to determine amino acid requirements (Sparks, 1998).

Isotopic infusion techniques

Contemporarily, isotopic tracers have been used in multiple ways to assess amino acid utilization and requirements. Isotope tracers can be used to measure amino acid oxidation both directly and indirectly. Generally, tracers are labeled with a stable isotope, however, radioactive tracers have been used as well (Stellaard and Elzinga, 2005). Because amino acids fed in excess of the amino acid's requirement, or above the level needed to support a given rate of protein synthesis, cannot be stored, surplus amino acids are catabolized. The rationale behind this method is based on the principal that when amino acids are metabolized and oxidized, whether it be due to requirement fulfillment or protein synthesis being limited, tracers labeled with isotopic carbon become destined for exhalation as carbon dioxide (Spahr et al., 2003). Labeled exhaled carbon dioxide can be measured as either $^{14}CO_2$ when radioactive isotopes are used or as $^{13}CO_2$ when stable isotopes are used. This concept allows for the use of direct amino acid oxidation and indicator amino acid oxidation methods. To test the oxidation of an amino acid directly, the amino acid whose requirement is under investigation is isotopically labeled. The test amino acid is fed at increasing levels until the requirement is reached, at which point measures of oxidation, such as breath $^{13}CO_2$ output, increase. The benefit of this method is that the oxidation of the test amino acid can be measured directly. The indicator amino acid oxidation method differs because the amino acid that is isotopically labeled is an

indispensable amino acid that is not the test amino acid. The oxidation of the isotopically labeled "indicator" amino acid is measured as the intakes of the test amino acid change. As the intake of the test amino acid increases from deficient to surplus, more of the indicator amino acid is able to be used for protein synthesis. Because the test amino acid becomes less limiting as intake increases, the amount of oxidation, and subsequent production of labeled CO_2, decrease. Once the requirement is fulfilled, a plateau in the oxidation curve is reached, and the amino acid recommendation can be obtained from a break-point analysis. The benefit of this method is that it is more versatile because certain amino acids are not directly converted to CO_2, therefore their requirements cannot be studied by using direct oxidation. These methods are particularly useful because they require shorter adaptations to diets due to their sensitivity to amino acid intake and rapid adaptation time shown in other species such as humans, poultry, swine and fish (Elango et al., 2008).

Dietary protein sources

Dietary protein sources commonly fed to horses have different protein contents and amino acid profiles. For example, forages commonly fed to horses, legume or grass hays, contain varying amounts of protein, and thus different amino acid profiles. Legume hays contain an average range of 18.7-23.9% CP, whereas grass hays contain an average range of 7.0-14.8% CP (Equi-Analytical, 2018). It is important to note that for grass hays that have been fertilized, these ranges of crude protein content do not account for non-protein nitrogen, which is not able to be used by horses. Many different factors can contribute to the protein

content and digestibility of forages, such as forage species, variety, plant maturity, leaf to stem ratio, and growing, harvesting and storage conditions. It has been well documented that across a growing season, legume species generally have higher protein contents than grass species and that the CP content decreases with plant maturity (Whitman et al., 1951; Darlington et al., 1968; Balde et al., 1993). It is also important to remember that these measures provide only the nitrogen content of the hay, and no information regarding the amino acid profiles of the hay. The digestibility of forage depends on multiple conditions, one of which being forage type, such as legume, grass or a mixture of the two types. Legumes and grasses, although both forages, differ with respect to chemical composition (Darlington et al., 1968; Särkijärvi et al., 2012). Grasses typically have a greater concentration of fiber and particularly neutral detergent fiber, such as cellulose, hemicelluloses and lignin, because legumes contain lower levels of hemicellulose (Van Soest, 1994; Wilman and Rezvani Moghaddam, 1998). Although these fiber sources cannot be digested directly by the horse itself, they are able to be digested by the microbial population within the horse. When analyzing grasses at the same maturity of legumes, typically more immature legumes are more digestible, however the nutritive value, voluntary intake and digestibility of alfalfa have been observed to wane with increasing maturity (Darlington et al., 1968; Fonnesbeck et al., 1967). Differences in growing conditions, such as soil nutrient content, temperature and rainfall, can alter forage growth and can also affect digestibility.

When comparing forages and concentrates, total digestible nutrients (TDN), which is the sum of crude protein, ether extract, nitrogen-free extract and

crude fiber within a feed, there are differences. For example, on average legume hays have a TDN of 60%, where grass hays have a TDN of 46% (Equi-Analytical, 2018). These values are much lower than soybean meal or corn for example, which have a TDN of 82% and 88% respectively (Equi-Analytical, 2018). It is important to note that while the calculations that comprise TDN use crude measures of nutrients, this measure does provide an estimate of value for the energy-producing components of feeds to the animal.

Concentrates commonly used in commercial feeds are soybean meal, soybean hulls, barley, oats, and corn, which also differ in protein content. Soybean meal contains on average of 46.3-55.8% CP, whereas soybean hulls contain on average 7.8-19.0% CP (Equi-Analytical, 2018). Dry barley has an average range of 9.7-14.2 % CP, dry oats have an average of 10.4-14.5% CP and dry shelled corn has an average of 7.5-10.5 % CP (Equi-Analytical, 2018). Again, it is important to remember that these measures do not provide the amino acid profiles of the different grains. Differences in digestibility exist between different grains as well, and the processing methods used on a type of grain can alter digestibility too (Rosenfeld and Austbø, 2008).

Protein Digestibility

Protein digestibility measures the amount of protein absorbed by the gastrointestinal tract, by comparing protein intake to fecal output. Understanding how efficiently a feed ingredient is digested aids in diet formulation, allowing for dietary protein levels in diets to be optimized to meet physiological requirements. The digestibility of nutrients can be calculated using the following formula:

$$\text{Digestibility } \left(\frac{\text{g}}{\text{kg}}\right) = \frac{\text{Nutrient intake (kg)} - \text{Fecal nutrient output (kg)}}{\text{Nutrient in feed (kg)}}$$

Digestibility of horse diets has been reported in two different ways: true and apparent digestibility. True digestibility corrects for endogenous losses, such as sloughed off intestinal cells or digestive secretions, whereas apparent digestibility does not (Gibbs et al., 1988). Digestion is distinguished as either pre-cecally or post-cecally, or post-ileally, depending on site of cannulation. Pre-cecally, enzymatic digestion predominates in the foregut, whereas post-cecally, microbial digestion occurs primarily in the hindgut. A study using ponies fitted with permanent ileal cannulas was conducted to detect and estimate differences in apparent and true digestibility, both pre-cecally and post-ileal, of grass and legume hays (Gibbs et al., 1988). The primary focus of this study was to detect differences between hay protein digestion in different segments of the gastrointestinal tract, because previous research provided mixed conclusions. Previously, it had been reported that protein digestion in the horse's large intestine was significant (Reitnour et al. 1969). However, one year later, pre-cecal protein digestion and absorption was suggested to be the major contributor to dietary protein metabolism in horses (Reitnour et al. 1970). In ponies, the major site of protein digestion was found to be the small intestine and the major sites of NDF digestion were in the colon and cecum (Hintz et al., 1971a). However, it was concluded that as forage intake increased, the relative importance of digestion from the hindgut also increased (Hintz et al., 1971a). Studying nitrogen partitioning along the digestive tract using digesta contents from 12 mature horses fed oats, corn and timothy revealed that the jejunum, ileum, cecum and small

colon are all major sites of net nitrogen absorption (Glade, 1983). From this study, only 20% of nitrogen disappearance was found to occur in the small intestine (Glade, 1983). This conclusion was supported when Gibbs et al. (1998) found that the legume hay with the greatest protein content had the greatest apparent pre-cecal nitrogen digestibility (21%) in ponies when compared to the other forages studied. Relative to the total tract nitrogen digestibility, the high-protein alfalfa hay was digested 28.5% in the foregut, and 71.5% in the hindgut, which was the greatest extent of foregut digestion for all the hays studied (Gibbs et al., 1988). Through regression analysis, it was determined that as nitrogen intake increased, apparent total tract nitrogen digestibility significantly increased from 13.4% for the coastal bermudagrass hay, to 19.7% for the low-protein alfalfa hay and finally 26.4% for the high-protein alfalfa hay (Gibbs et al., 1988). When comparing the regressions of apparent pre-cecal digestibility to apparent post-ileal digestibility of ponies fed solely-forage diets, it is clear that the majority of the nitrogen digestion occurred post-ileally (Gibbs et al., 1988). This is consistent with other literature concluding that the major portion of forage is digested in the hindgut (Gibbs et al., 1996; Miyaji et al., 2008). Interestingly, when ponies consumed the high-quality legume, the apparent pre-cecal digestibility increased (Gibbs et al., 1988). Estimates of true digestibility indicated that dietary nitrogen digestion was almost complete when looking at total tract digestibility of the average of the three forages studied (Gibbs et al., 1988). Using regression equations from data averages, true pre-cecal nitrogen digestibility was estimated to be 37%, whereas true post-ileal nitrogen digestibility was estimated to be 96%

(Gibbs et al., 1988). The regression equation was not statistically significant and had a low r^2, which is likely due to high variation, possible due to low sample numbers obtained in this study,

When three mature ponies fitted with ileal cannulas were fed different cereal grains and oilseeds, pre-cecal, post-ileal and total tract digestibility of nitrogen was able to be quantified (Gibbs et al., 1996). Ponies were arranged in two 3x3 Latin squares for two trials and were supplied with 25% of their daily ration as coastal Bermuda grass hay (Gibbs et al., 1996). The first trial used corn, oats and sorghum as dietary treatments. The second trial used a basal ration of 100% hay, hay supplemented with soybean meal and hay supplemented with cottonseed meal as dietary treatments (Gibbs et al., 1996). Results from the first trial revealed no differences in the percentages of apparent pre-cecal, post-ileal or total tract nitrogen digestibility between the three cereal grains studied (Gibbs et al., 1996). The basal diet of hay in the second trial was not different with respect to pre-cecal (31%), post-ileal (77%) or total tract (84%) apparent digestibility when compared to hay diets supplemented with either soybean meal or cottonseed meal (Gibbs et al., 1996). In previously conducted research, apparent pre-cecal nitrogen digestibility of soybean meal in horses has been seen to range from 58 – 72% (Farley et al., 1995). Gibbs et al. (1996) found the apparent pre-cecal digestion of soybean meal and coastal Bermuda grass hay to be 42%. The relative digestion of pre-cecal to total tract digestibility of the all-hay diet was 37%, which was significantly lower than when the hay was supplemented with soybean (48%) or cottonseed meal (67%) (Gibbs et al., 1996). From these findings, the addition

of forage appears to reduce the pre-cecal digestibility of cereal grains, namely soybean meal.

Differences in protein digestibility of processed cereal grains, oats, barley and maize, have also been characterized by Rosenfeld and Austbø (2008). Using four mature, cannulated horses, 16-20 nylon mobile bags were filled with samples of each of three different feed types (oats, barley and maize) that were processed in four different ways (ground, pelleted, extruded and micronized) and were nasogastrically intubated into the stomach to simulate digestion (Rosenfeld and Austbø, 2008). The pre-cecal protein digestibility of oats (68%) was found to be greater than when horses were fed barley (59%), but not different from when horses consumed maize (62%) (Rosenfeld and Austbø, 2008). Total tract protein digestibility however was lowest when horses consumed oats (75%) and not different when horses consumed either barley (83%) or maize (84%) (Rosenfeld and Austbø, 2008). By calculating the difference of total digestibility and pre-cecal digestibility, it was observed that the relative post-cecal to total tract digestibility of oats, barley and maize were 9.6%, 28.6% and 27% respectively. This supports that the majority of cereal grains were digested pre-cecally. Pre-cecally, pelleted grains (68%) were significantly more digestible than extruded grains (56%) on average ($P = 0.046$; Rosenfeld and Austbø, 2008). With respect to total tract digestion, micronized grains (82%) were significantly more digestible than ground grains (79%) ($P = 0.006$; Rosenfeld and Austbø, 2008). By calculating the difference of total digestibility and pre-cecal digestibility, it was observed that the relative post-cecal to total tract digestibility of ground, pelleted,

extruded and micronized grains were 21.4%, 15.2%, 31.0% and 20.8%, respectively. A common limitation of these digestibility studies is that the individual amino acid digestibilities of each type of grain or forage were not characterized.

In a single study by Almeida et al. (1999), five foals were fitted with an ileal cannula to determine the prececal digestibility of amino acids in diets commonly fed to horses. Foals were fed five levels of crude protein and received half of their daily intake as concentrate, and the other half as mixed grass hay (Almeida et al. 1999). It was recorded that apparent pre-cecal digestibility of lysine ranged from 52-69% and 32-62% for threonine (Almeida et al. 1999). A limitation of this study is that it used corn and soybean meal, and not just one feed ingredient, which prevents comparisons to ileal digestibilities in other species. Future research directions should include investigating the amino acid digestibilities of one single feed ingredient in horses.

Protein feeding practices in the equine industry

Over the last few decades, feeding practices of horses have been surveyed and results from several studies agreed that horses were being fed protein well in excess of their requirements. Research regarding protein, energy and dry matter consumptions of equine athletes was conducted by Gallagher et al. (1992b and 1992b) to determine feeding practices in the racing industry. Their first study revealed that Thoroughbred race horses were fed an average of 126% of the 1989 NRC recommendations for crude protein intake (Gallagher et al., 1992a). Results from their second study showed that racing Standardbreds were similarly fed an

average of 127% of the 1989 NRC recommendations for crude protein intake (Gallagher et al., 1992b). In North Carolina, a regional survey of 50 riding horse owners found that on average 70% of horses were provided crude protein in excess of the 1989 NRC recommendations for crude protein intake (Honore and Uhlinger, 1994). A survey comparing industry feeding practices used eleven horse farms encompassing a total of 201 horses within the Chesapeake Bay Watershed to evaluate the 2007 NRC's protein requirements (Harper et al., 2009). On average, horses were fed 157% of the NRC's recommendation for crude protein and the high end of the range was 263% (Harper et al., 2009).

Horses cannot store amino acids in the body above the level needed to support protein synthesis and other metabolic functions. When amino acids are fed in excess of requirements, or when feed is consumed that is deficient in limiting amino acids, those surplus amino acids that cannot be used by the body are catabolized and destined for excretion. During amino acid catabolism, the carbon backbones are ultimately converted to CO_2 and destined for excretion via exhalation. The amino moieties enter the urea cycle in the liver and are ultimately converted to urea and destined for excretion in urine. Furthermore, undigested dietary protein is excreted in the feces. Nitrogen excretion into the environment can have numerous environmental and health implications.

Environmental implications of overfeeding protein

Nitrogen excreted in the urine and feces pollutes the air, land and water and contributes towards aerosol formation, soil acidity and eutrophication (Chowdhury, 2013; Nixon, 1995). Animal excrement in pastures can leach from

the soil during rain fall and pollute nearby waterways, which can be detrimental to aquaculture (Carpenter et al., 1998). When urinary urea is broken down by bacterial enzymes, which occurs when urine and feces come in contact with each other, ammonia volatilization can occur. The greatest source of ammonia emissions in the United States come from animal agriculture industries, however knowledge regarding the equine industry's contribution is currently limited (USEPA, 2005). From the limited information available, the Environmental Protection Act (EPA) proclaimed from a report in 2002 that only 2.9% of total ammonia emissions from animal husbandry operations in the United States derive from the equine industry (EPA, 2005). Recently it has been determined that feeding sedentary mature horses at various levels above the 2007 NRC's crude protein requirements results in an increase in NH_3 emissions from both urine and feces (Weir et al., 2017). Given that a horse's lifespan may be upwards of 25-30 years, and that the majority of other livestock species in animal husbandry operations are not permitted to live quite as long, it is possible the impact from the horse industry is underestimated. Regardless of the proportion of the horse industry's contribution, adverse effects on animal and caretaker health still exist. It has been recently reviewed in cattle that large concentrations of volatilized ammonia have the ability to reduce atmospheric visibility, as well as impair animal and human health (Hristov et al., 2011).

Health implications of over-feeding protein

Inhalation

Inhaling high levels of ammonia is known to cause respiratory tract inflammation and can cause loss of lung function in humans (Arwood et al., 1985; Crook et al., 1991), and horses (Katayama et al., 1995). Ammonia pollution generates a noxious gas, which can irritate the eyes and respiratory tracts of humans and animals alike (Pickrell, 1991). Ammonia inhalation can also exacerbate symptoms of chronic obstructive pulmonary disease (COPD) in horses (Vandenput et al., 1998). When comparing equine stables to pastures, ambient ammonia levels were reportedly100-200 ppb higher in stables (Whittaker et al., 2009). Ammonium hydroxide ($NaOH_4$), a strong alkali, forms when ammonia reacts with water in the mucosa, which could alter breath pH. Stabled ponies had an average breath pH of 4.8, which was found to be significantly greater when compared to pastured ponies who had an average breath pH of 4.5 (Whittaker et al., 2009). This is problematic not only for horses housed in stables for long periods of time, but also for animal caretakers as well. A short-term exposure limit for ammonia of 15 minutes at 35 ppm for humans has been defined by the Center for Disease Control (CDC) (CDC, 2011). Some reports of air ammonia concentrations in equine stables have been deemed unsafe by the CDC (Bott et al., 2015).

Water Loss

When excess amino acids are catabolized, transamination reactions increase and urea is formed to help clear blood levels of excess ammonia. Because urea must be dissolved in water in order to be excreted, as excess amino acid intake increases and urea formation increases, water intake and urine output

also increase (Connysson et al., 2006; Funaba et al., 1996; Graham-Thiers et al., 2001; Kim et al., 2011). Recently, it was reported that differences in dietary crude protein concentration did not affect water intake, but that urinary output increased by 5.5% when dietary crude protein concentrations increased in horses (Oliveira et al., 2015). Increased urination rates without an increase in daily water intake can lead to dehydration over time, which is detrimental particularly to horses in moderate to intense work or those living in hot climates. When evaluating the consumption and excretion of water by horses consuming different species of legume and grass hays, a greater volume of excreted urine was reported when horses consumed the legume forages (Fonnesbeck, 1968). Interestingly, due to the increased digestibility of legume forages compared to grass forages, when horses consumed legumes more water was able to be absorbed through the gastrointestinal tract and thus there was less water excreted in the feces (Fonnesbeck, 1968).

Calcium Metabolism

The literature regarding the effects of excess protein intake on calcium metabolism is inconsistent. When amino acid catabolism and urine output increase, it has been reported in humans that calcium retention decreases, as reviewed by Delimaris (2013), Heaney (2002) and Roughead (2003). During this metabolic process, calcium concentrations in the urine have also been seen to increase in cats and humans (Hashimoto et al., 1996; Heaney, 2002; Roughead, 2003). In adults, protein consumption above the dietary recommendations, which is greater than 0.8 g protein/kg BW/d or about 142 g/d, resulted in disorders of

bone maintenance, calcium metabolism, renal and liver function as reviewed by Delimaris (2013). In horses, high protein intake has been shown to increase urinary calcium concentrations (Glade et al., 1985).

Alternatively, it has been reported that excessive protein intake does not appear to adversely affect calcium metabolism, or the musculoskeletal development of growing horses (Boren et a1 1987; Cymbaluk et al., 1990; Schryver et a1 1987). There is evidence suggesting that ingesting high concentrations of dietary amino acids, other than methionine and cystine, may be capable of improving bone health (Gaffney-Stomberg et al., 2010). Clearly, there are discrepancies regarding differences in calcium metabolism when horses ingest excess protein and more research is needed to discern the nature of these effects.

Heat Production and Energy Expenditure

Using increased protein in conjunction with low carbohydrate intake is the premise of many diets, including the Adkins® diet. It has been well established in the literature that there is an energy cost associated with using protein as a primary fuel source. In the 19th century, the Atwater system developed equations to estimate the availability of energy in carbohydrates, protein and fat from dietary sources. On average, it was estimated that the heat of combustion, or the cost of catabolism, for 1 gram of carbohydrates, protein and fat were respectively 4.18, 5.65 and 9.44 calories. The thought process is that, the more complex a molecule is, a greater heat of combustion is required for catabolism. Due to both the inefficiency of catabolizing amino acids and the energetic cost associated with urea synthesis, heat produced from using protein as an energy source is greater

when compared to using other substrates (Belko et al., 1986). In swine, excess protein has been correlated to increased heat production (Kerr et al., 2003). As reviewed by Yamaoka (2008), humans have been intravenously administered amino acids during to prevent hypothermia Although this has been studied in other species, particularly humans, the magnitude of these effects is currently not well studied in horses. One study has been conducted analyzing the plasma and muscle amino acid composition of six horses in racing condition fed either the recommended (12.5%) or a high (16.6%) dietary protein grass silage (Essen-Gustavsson et al. 2010). The silages were produced in the same area, but the levels of nitrogen fertilizer and cutting time were altered to create a similar silage with different levels of crude protein and digestible energy content (Essen-Gustavsson et al. 2010). Despite the high protein group consuming considerably more energy than the low protein group, there were no differences in weight gain between the groups. This could be evidence of increasing metabolic costs of metabolizing excess protein, which has been discussed by Connysson et al. (2006).

Cost implications of overfeeding protein

Historically, the cost of feed has been a considerable expense of owning and maintaining horses (Cooper, 1917; Harris, 1999; Langdon, 1982; Łojek et al., 2009). In commercially made horse feeds, it is common to see that as the value of protein increases, so does the price. It has been reported that as the relative feed value and crude protein content of alfalfa hay increases, so does the price of the hay (Canbolat et al., 2006). These costs can be mitigated by using the ideal

protein concept in diet formulation and feeding practices. Unfortunately, more research needs to be conducted to clearly define the amino acid requirements of horses before this principal can be implemented.

Reducing nitrogen excretion in horses

Some studies have been conducted demonstrating that a reduction in total crude protein intake may limit protein synthesis and growth in horses (Jordan and Myers, 1972; Ott et al., 1979a; Gibbs et al., 1989; Saastamoinen et al., 1994; Tanner et al., 2014). Recently, it has been reported that nitrogen excretion in horses can be reduced without compromising growth parameters or muscle composition. Using Italian heavy draft horses raised for slaughter, a study was conducted measuring effects of carcass traits in response to a reduction in crude protein intake (Mantovani et al., 2014). This study was completed in two phases to observe differences in age. To do this, for the first phase only two levels of protein were fed to half of the horses, then half of the horses consuming the high protein and half of the horses consuming the low protein were euthanized, while the remaining horses continued to the second phase of the study. Both the high and low protein feeds in the second phase had increased protein to account for differences in protein requirements as the horses aged. Therefore, horses that continued to the second phase were studied consuming a total of four levels of crude protein. Horses in phase one were fed either a 10.6% and a 13.2% CP feed and were aged 276 ± 35 days at the beginning of the phase one. The remaining horses in phase two were 424 ± 26 days at the beginning, and were fed either a 11.2% or 14.7% CP feed. No differences were seen when comparing growth,

carcass weight and dressing percentages of horses fed diets only 3% different in crude protein content. As horses age throughout the first years of their lives, their crude protein requirements wane on a body weight basis if exercise does not increase requirements. When horses were examined between 424 and 533 days of age, it was seen that there was a significant effect of dietary protein reduction on levels of blood glucose and urea nitrogen concentration (Mantovani et al., 2014). However, a limitation of this study is that horses were fed in lots in groups, so individual daily intake was not able to be computed. It was noted that some horses were excluded from the trial due to being victims of aggressive behavior. Differences in dietary treatment effect could have been a result of intakes differing due to group feeding behavior. This study is also unique because typically horses are not raised for slaughter, unlike other species. More research is needed to determine the effects of reducing crude protein intake, particularly in sedentary mature horses.

Reducing nitrogen excretion in other livestock species

Within the animal food industry research has shown that it is possible to successfully reduce nitrogen excretion without effecting growth or performance parameters. Many studies have been conducted in the swine (Girotto et al., 2013; Shriver et al., 2003), poultry (Ospina-Rojas et al., 2012; Summers, 1993), dairy (Groff and Wu, 2005) and fish (Cheng et al., 2003; Lima et al., 2015) industries to reduce crude protein intake and without altering production. Some methods include modifying the sources of ingredients to change the protein contents of feed, supplementing diets with indispensable amino acids and targeting amino

acid requirements to optimize feed provisions. Proper knowledge of the limiting amino acids, as well as the individual amino acid requirements, are necessary to achieve the concept of ideal protein. Implementing the findings of this research will help reduce the environmental footprint of the livestock industry, mitigate fiscal losses, optimize growth and uphold animal and caretaker health.

Conclusion

Research regarding limiting amino acids and achieving the ideal protein concept in horses is limited. As a result, current protein feeding practices of horses do not place enough emphasis on amino acid adequacy. Although there have been attempts at determining limiting amino acids in horses, lysine levels in growing horses is the only area of research that has received sufficient attention. Research using multiple methods and criterion is needed to determine dietary amino acid adequacy, as well as amino acid requirements, in order to implement the 'ideal protein' concept in dietary formulation. Optimizing how amino acids are fed to horses will reduce nitrogen losses to the environment and will positively impact the air quality and horse health, as well as those mucking stalls, and mitigate fiscal losses. Being that the typical diet of horses is predominantly forage-based, understanding the ability of forage-based diets to meet whole-body protein requirements and identifying potentially limiting amino acids is of the upmost importance.

Chapter 3: Rationale and objectives of the reported studies

Additional research is needed to determine the ability of predominantly forage diets to provide adequate amounts of limiting amino acids in growing and mature horses. There is an opportunity for the equine industry to apply methodologies validated in other species to determine if predominantly forage diets can meet horses' daily amino acid requirements. The importance of understanding how measures of whole-body protein metabolism are altered by diet in the sedentary mature and growing horse, coupled with the lack of data pertaining to these measures in the horse, merits research in these areas. Forage is often fed with a ration balancer supplement to help meet vitamin, mineral and amino acid requirements, without providing too many excess calories. To help optimize protein synthesis, amino acid supplementation has been used successfully in other species, including horses. Understanding how well the amino acid profile supplied by forages meets the horse's requirements will allow for diet formulations to better optimize protein metabolism. Additionally, characterizing amino acid adequacy in forage-based equine diets will help to optimize dietary amino acid intake and hopefully reduce nitrogen waste in the long run. The objective of the following studies was to contribute towards the existing knowledge of protein and amino acid adequacy in both mature and growing horses fed predominantly forage diets. These studies are the first to use isotope kinetics, along with other measures, to evaluate the effects on measures of whole-body protein metabolism in horses receiving a predominantly forage diet

supplemented with potentially limiting amino acids in mature horses, and either a high or low protein ration balancer in growing horses.

Objective of Chapter 4:

To determine whether lysine, threonine or histidine supplementation to either a grass or legume forage-based diet would improve measures of whole-body protein synthesis in sedentary mature horse

Objective of Chapter 5:

To determine whether whole-body protein metabolism of growing horses would be altered when horses consumed either a grass or legume forage-based diet supplemented with either a high or low protein ration balancer.

Chapter 4: Effect of forage type and amino acid supplementation on measures of whole-body protein metabolism in mature horses consuming a predominantly forage diet

Abstract

All forage diets can fulfill caloric needs of horses at maintenance or light work; however, forage type influences protein content. Legume hays contain a higher crude protein percentage than grass hays. The objective of this study was to determine the effects of either timothy and alfalfa hay cubes supplemented with lysine, threonine or histidine on measures of whole-body protein metabolism. Eight mature Thoroughbred mares (aged 15 ± 3 years; 575 ± 33 kg, BW) were fed 2% of body weight per day of either a timothy (CP 10.1%) or alfalfa (CP 14.1%) hay cube treatments for 7 days. Horses were randomly allocated to receive one of five amino acid-supplemented diets: either glutamine ((-) Con), lysine, threonine, histidine or lysine, threonine and histidine ((+) Con). Horses were allocated to the different amino acid-supplemented diets such that all horses were studied on all treatments and no two horses were receiving the same treatment at the same time, using a randomized crossover design. After adapting to each treatment for 6 days, blood samples were collected by jugular venipuncture pre- and 90 minutes post feeding the morning meal. The blood plasma was analyzed for amino acid concentrations using HPLC and urea nitrogen concentrations using a spectrophotometric assay. On day 7, isotope infusion procedures included a 2h primed, constant intravenous infusion of [^{13}C]sodium bicarbonate to measure CO_2 production and a 4h primed, constant oral administration of [1-^{13}C]phenylalanine, for phenylalanine oxidation and estimated rates of non-oxidative phenylalanine disposal. Blood and breath samples were collected throughout the isotope infusion to measure plasma [^{13}C]phenylalanine enrichment, using GC-MS, and breath $^{13}CO_2$ enrichment, using an infrared isotope analyzer. Data were analyzed using PROC GLIMMIX procedure of SAS version 9.2, with treatment, time and the interaction between the two variables as the fixed effects. To Plasma urea nitrogen concentrations ($P < 0.0001$) and the amount of oxidized phenylalanine ($P = 0.0008$) were higher when horses consumed the alfalfa-based diets. Plasma amino acid concentrations of the amino acids supplemented, lysine ($P < 0.0001$), threonine ($P < 0.0001$) and histidine ($P < 0.0001$), were responsive to changes 90 minutes post-feeding. There were no apparent benefits to whole-body protein synthesis from any of the dietary amino acid supplements for any of the parameters studied. The amount of protein provided by the timothy hay cubes supported the same rate of whole-body protein synthesis as the alfalfa hay cube treatment. Mature horses at maintenance consuming forages with a higher crude protein percentage or additional amino acid supplementation derive no benefit with regards to whole-body protein synthesis.

Introduction

Forage is the foundation of the equine diet and is able to provide enough digestible energy and adequate crude protein for mature, sedentary or lightly exercised horses. Crude protein is an estimate of dietary protein based on the amount of nitrogen in the feed and is the gold standard used for formulating diets to meet protein requirements (NRC, 2007). Forages can be classified as grasses or legumes based on cell structure and the plant's ability to fix nitrogen. One of the big differences between legumes and grasses is that legumes form a symbiotic relationship with nitrogen-fixing bacteria called rhizobium, which provides more nitrogen for the plant and thus legumes have greater crude protein contents. However, not all dietary protein sources are composed of the same amino acid profiles. Therefore, rather than having a total crude protein requirement, horses have a physiological requirement for each of the indispensable amino acids, those that cannot be made *de novo*, as well as a requirement for amino nitrogen and carbon backbones. Unfortunately, there is limited information regarding the indispensable amino acid requirements of horses, with only the lysine requirement being provided by the NRC (NRC, 2007). To ensure that adequate levels of digestible energy are provided by the diet, often dietary crude protein intake exceeds requirements. Without knowledge of horses amino acid requirements, nitrogen is typically fed in excess of its requirement to ensure that adequate levels of indispensable amino acids are provided (Gallagher et al., 1992a; Gallagher et al., 1992b; Honoré and Uhlinger, 1994; Harper et al., 2009).

Amino acids provided in excess of requirements cannot be stored by the body, so when horses consume nitrogen above their requirements, excess amino acids need to be metabolized by the body. When catabolized, the nitrogen component of amino acids ends up as ammonia and ultimately urea, which is then excreted in the urine. Once excreted, if the urine comes into contact with the fecal microbes, it may be metabolized back to ammonia and excess environmental ammonia can have detrimental health and environmental effects (Bussink and Oenema, 1998). These outcomes can be mitigated by reducing excess nitrogen intake, more specifically, by meeting the requirement for the indispensable amino acids as closely as possible. However, research regarding amino acid requirements in horses and the potentially limiting amino acids in mature horses is limited.

Protein synthesis becomes limited when an indispensable amino acid is provided below its requirement, and this amino acid is termed a limiting amino acid. Previous studies have investigated potentially limiting amino acids in mature horse diets. Lysine, for example, has been established as the first limiting amino acid in growing horses (Ott and Asquith, 1986). In growing horses, lysine supplementation increased growth, as well as the feed to gain ratio (Hintz et al., 1971b). In mature and aged horses supplemented with lysine and threonine, an increase in a subjective muscle mass scores and decrease plasma urea nitrogen has been shown (Graham-Thiers and Kronfeld, 2005). Some studies have suggested that threonine is the second most limiting amino acid in a typical equine diet (Graham et al., 1994; Staniar et al., 2001; Graham-Thiers and

Kronfeld, 2005; Tanner et al., 2014; Yoshida and Ohta, 2018). Histidine has also been suggested as a potentially limiting amino acid in diets typical to lactating cattle and weanling horses (Vanhatalo et al., 1999; Tanner et al., 2014); however, histidine supplementation has not been previously studied in horses. For these reasons, the supplementation of lysine, threonine and histidine to forage-based diets were of interest in mature horses at maintenance.

Several methods can be used to measure whole-body protein metabolism in horses, some of which include the use of isotopic tracers. These methods are based on the principle that amino acids that are not used in protein synthesis, or to synthesize other metabolites, are oxidized (Allison, 1955). When amino acids are fed in excess of requirements, the excess amino acids are destined for catabolism and subsequent oxidation. The tracers are labeled with isotopic carbon that is exhaled in the form of carbon dioxide, which can be measured and analyzed (Kingdon et al., 2000; Spahr et al., 2003). Isotope infusion methods also allow estimates of the rate of protein synthesis by directly measuring the rate of oxidation of an isotopically-labeled indicator amino acid. The stable isotope of a non-limiting amino acid is infused at a constant rate and the isotope enrichment of periodic blood and breath samples is used to calculate its rate of oxidation. When calculating the difference between the flux and oxidation, rate of protein synthesis can be obtained.

Isotopic methods were initially used in growing pigs to determine amino acid and total protein requirements (Elango et al., 2007; Elango et al., 2011; Humayum et al., 2007a; Kim et al., 1983; Kurpad et al., 2003; Lazaris-Brunner et

al., 1998; Moehn et al., 2008; Wilson et al., 2000; Zello et al., 1993), the metabolic availability of amino acids (Humayun et al., 2007b; Moehn et al., 2005), and to determine limiting amino acids (Brunton et al., 2007). Isotope infusion methods have also been used in horses to study amino acid requirements and dietary amino acid adequacy (Urschel et al., 2012; Mastellar et al., 2016b; Mok et al., 2018; Smith, 2016), as the effects of other factors such as age and disease on protein synthesis in horses (Wagner et al., 2013; Mastro et al., 2014). Therefore, the objective was to use isotopic methods and other measures of whole-body protein metabolism to determine whether lysine, threonine or histidine supplementation to either a grass or legume forage-based diet would result in improvements in measures of whole-body protein synthesis in mature horse at maintenance.

Materials and methods

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals, Housing, and Feeding

Eight Thoroughbred mares aged 15 ± 3 years (mean ± standard deviation) were obtained from the University of Kentucky Department of Animal and Food Sciences' research herd. Initially, horse's body weights ranged from 527 kg to 625.5 kg, with an average of 575 ± 33 kg. Horses were weighed using an electronic scale (TI-500, Transcell Technology Inc., Buffalo Grove, IL). Body condition scores ranged from 5 to 6 and horses maintained bodyweight throughout the duration of the study (Henneke body condition scoring scale).

They were all housed individually in 3.7 × 3.7 m stalls overnight. Between the hours of 0800 and 1500, horses were turned out in a 2.5-acre paddock and fitted with muzzles to prevent grazing. Horses were fed their experimental diets at 0700 and 1500, and any feed refusals were collected after the morning meal and weighed each day.

Experimental Design

Horses were randomly allocated into two groups and fed one of ten treatments in a randomized crossover design. Horses were first allocated to one of two different forage types (alfalfa hay cubes to represent a legume species and timothy hay cubes to represent a grass species), then within each forage type, five combinations of either different amino acids or amino acid mixtures were added to a fixed amount of oats, as described below. Horses were adapted to their respective forage, housing and handling procedures for 7 days prior to the beginning of period 1. On day 0 of each period, horses were fed their experimental diet in the morning and continued to consume that diet for 7 days. On day 6, pre- and 90 min post- feeding blood samples were taken to determine plasma urea nitrogen and amino acid concentrations, and on day 7, whole body phenylalanine kinetics were studied using stable isotope methodologies. These procedures will be outlined in greater detail below. At the end of the day 7 sampling procedures, horses were then switched to the next dietary treatment on the afternoon of day 7 after the isotope administration procedures were completed. Once they had completed all five amino acid supplements within their initial forage type, they were switched over to the second forage over a seven-day

period before the next treatment period began. All treatment combinations and procedures were repeated for each forage type in every horse.

Dietary Treatments

The experimental diets were formulated to meet, or exceed, the estimated requirements for digestible energy, crude protein, and indispensable amino acids (Table 4.2; NRC, 2007). Either alfalfa hay cubes or timothy hay cubes (Southern States Cooperative, Richmond, VA), depending on the treatment allocation, were offered at 2.0% of bodyweight on an as-fed basis. All horses, regardless of treatment allocation were fed 1kg of oats (Southern States Cooperative, Richmond, VA) supplemented with either an assigned amino acid or amino acid combination and mixed with a mineral premix (Alfa Plus™ and Grass Plus™ Buckeye Feeds, Dayton, OH). The mineral premix provided in the dietary treatment corresponded to either timothy or alfalfa to compensate for any forage-specific lack of minerals. Daily hay cube, oat and amino acid supplement allocations were divided evenly between the two daily meals. For each forage type, there were 5 treatments: glutamate ((-) Con), lysine, threonine, histidine, and lysine + threonine + histidine ((+) Con). Glutamate was provided as L-glutamic acid (Sigma Aldrich, St. Louis, MO), histidine was provided as L-histidine monohydrate HCl (J.T. Baker, Phillipsburg, NJ) lysine was provided as L-lysine HCl and threonine was provided as L-threonine (Ajinomoto Heartland, Inc., Chicago, IL). This resulted in a total of 10 treatments: alfalfa and (-) Con, alfalfa and lysine, alfalfa and threonine, alfalfa and histidine, alfalfa and (+) Con, timothy and (-) Con, timothy and lysine, timothy and threonine, timothy and

histidine, and timothy and (+) Con. Horses were allocated to the different amino acid-supplemented diets such that no horse received the same amino acid-supplemented diet twice.

The amount of each amino acid added to the treatments was determined based on the daily lysine requirement estimated by the NRC (2007), 55 mg/kg/d for a mature, idle horse. Using the lysine requirement, the daily requirements of threonine and histidine were estimated to be 34 and 32 mg/kg/d, respectively, based on the ratio of lysine to these amino acids in the muscle (NRC, 2007). Providing the supplemental amino acids at these levels ensured that in combination with the amounts provided by the forage, levels were provided well above the estimated requirement and so if any of these amino acids were limiting in the forage source, responses should have been seen in the measured variables. In order to ensure that any differences in responses between amino acid treatments, within a forage type, that occurred were due to the targeted amino acid supplementation, and not simply added amino nitrogen, glutamate was used to ensure that all treatments were isonitrogenous. A similar strategy to calculating the amounts of supplemental amino acids has been used previously in horses (Urschel et al., 2012). Therefore, the lysine treatments were supplemented with the daily recommended 55 mg/kg/d lysine (fed as 67 mg/kg/d lysine HCl) and 72 mg/kg/d glutamate; the threonine treatments were supplemented with 34 mg/kg/d threonine and 141 mg/kg/d glutamate; the histidine treatments were supplemented with 32 mg/kg/d histidine (fed as 43 mg/kg/d histidine monohydrate HCl) and 153 mg/kg/d glutamate; the (+) Con treatments provided 55 mg/kg/d lysine (fed

as 67 mg/kg/d lysine HCl), 34 mg/kg/d threonine and 32 mg/kg/d histidine (fed as 43 mg/kg/d histidine monohydrate HCl); and the (-) Con treatments provided 183 mg/kg/d glutamate).

To ensure that the vitamin and mineral contents of diets met estimated requirements, vitamin/mineral premixes, which were specific to either the alfalfa or timothy cubes, were top dressed on the oats (Alfa Plus™ and Grass Plus™ Buckeye Feeds, Dayton, OH). Horses being fed alfalfa hay cubes were fed the alfalfa vitamin/mineral mix at 170 mg/kg/d and horses being fed timothy hay cubes were fed the grass mineral mix at 190 mg/kg/d. The analyzed nutrient compositions of the oats, timothy and alfalfa hay cubes are shown in Table 4.1.

Sampling and isotope infusion

All animals were weighed on day 6 of each experimental period, prior to pre-feeding blood collection. Blood samples were collected on day 6 of each study period prior to the morning meal and 90 postprandial to measure the effects of amino acid supplementation on plasma amino acid and plasma urea nitrogen concentrations. Blood was collected via venipuncture from the jugular vein into heparinized vacutainers (BD, Franklin Lakes, NJ) and immediately centrifuged at 1,500 × g for 10 minutes at 4 ºC. The supernatant plasma was collected and stored at -20 ºC until the time of analysis.

On the morning of day 7, one jugular intravenous catheter (14G, 9 cm) was aseptically fitted for isotope infusion. Whole-body phenylalanine kinetics were determined using a 2 h primed, constant intravenous infusion of [^{13}C] sodium bicarbonate at a prime rate of 5.3 μmol/(kg BW) and a constant rate of 4.4

μmol/(kg BW·h) followed by a 4 h primed, constant oral administration of [$^{1\text{-}13}$C] phenylalanine at a prime rate of 10.23 μmol/kg and a constant rate of 7.22 μmol/(kg BW·h) (Cambridge Isotope Laboratories, Andover, MA), as previously described (Urschel, 2012). The prime to constant ratio (1.4:1) for the phenylalanine isotope was previously validated in horses (Urschel et al., 2012), and the dose of phenylalanine has been shown to result in stable, measurable plateaus of isotope within the breath (Mastellar et al., 2016a). The goal of bicarbonate isotope infusion was to estimate total CO_2 production, whereas the phenylalanine isotope administration allowed for the measurement of phenylalanine oxidation to CO_2 and phenylalanine flux, which enabled the subsequent estimates of rates of whole-body protein synthesis and degradation (Urschel et al., 2012).

In order to use steady-state isotope kinetics, frequent small meal feeding was done to ensure that horses were at a steady metabolic state during isotope infusions. At approximately 0700 horses were fed their first meal containing 3/48 of their daily allocation, 90 minutes before the start of the bicarbonate infusion. Thirty minutes after, 1/48th of their daily allocation was fed every 30 min throughout the duration of isotope infusion. Feed refusals were minimal, and any feed refusals that remained after 30 minutes were removed and weighed. Immediately prior to isotope infusion, two baseline blood and breath samples were collected. Blood was collected from a catheter placed in the jugular vein, centrifuged at 1,500 × g for 10 minutes at 4 ºC. The supernatant plasma was promptly collected and stored at -20 ºC until the time of analysis. Breath was

collected into gas impermeable bags using a modified equine Aeromask (BreathEazy Ltd, Malvern, Worcestershire) and immediately analyzed, as described below (Urschel et al., 2012). Following baseline sample collection, [^{13}C] sodium bicarbonate was infused into the intravenous catheter using a cordless infusion pump (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc.) attached to a surcingle worn by the horse. During the bicarbonate isotope infusion, breath samples were collected every 30 min for the first hour, then every 15 min during the second hour. After 2 h, infusion was stopped and horses received their [1-^{13}C] phenylalanine prime dose top dressed onto their meal, and the [1-^{13}C] phenylalanine was top dressed onto all remaining meals. Throughout phenylalanine administration, blood and breath samples were collected every half hour. At the end of the isotope administration period, the catheter was removed and horses received their day 0 meal for the next treatment period.

Sample Analysis

Pre- and post-feeding blood samples from day 6 were analyzed for plasma amino acid and urea nitrogen concentrations. A colorimetric spectrophotometric assay was used to measure plasma urea nitrogen concentrations. A 10-μL aliquot of each plasma sample was pipetted, in duplicate, into microcentrifuge tubes containing 125 μL of chilled urease buffer (Sigma-Aldrich Co., St. Louis, MO). The plasma samples were pipetted on the side of the tube, above the urease buffer, so that the enzymatic reaction for all samples was able to be initiated at the same time, using a multi-tube vortexer (Scientific Manufacturing Industries, Emeryville, CA). After 20 min of incubation in a rack on ice, 250 μL of phenol

nitroprusside solution was added to all tubes with a repeat pipetter, then vortexed. Next, 250 μL of alkaline hypochlorite solution, and 1000 μL of distilled water were added to each sample, then vortexed. After 25 min of incubation at room temperature, 200 μL of each sample was transferred in duplicate to 96 well plates, placed in a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA,) and read at 570 nm of wavelength. The inter-assay variation between plates for the control sample was 8.56% and the average intra-assay variation within each plate for the control sample was 3.43%.

Using high performance liquid chromatography (HPLC) with a previously described method (Bidlingmeyer et al., 1984; Urschel et al., 2011), plasma amino acid concentrations were determined. Norleucine was added to all samples as an internal standard, then all samples were deproteinated using 10-kd cutoff centrifugal filters spun at 15,000 x g for 30 minutes at 4 ºC, and the filtrate was freeze-dried. A re-dry solution of methanol, 1mM of sodium acetate, and trimethylamine (TEA) (Fisher Scientific, Fair Lawn, NJ) were combined in a 2:2:1 ratio, and vortexed. A 10uL aliquot of re-dry solution was added to all tubes, then 25uL of a 0.2mM glutamine solution was added to standards, then all tubes were re-freeze dried. Amino acids were then derivatized by adding 20 μL of the derivatizing solution comprised of methanol, TEA, water, and phenylisothiocyanate (Acro Organics, Geel, Belgium) in a 7:1:1:1 ratio and incubating the samples in a sealed container at room temperature for 20 min. Samples were then freeze-dried and reconstituted with 100 uL of HPLC eluent

and injected onto a 3.9x300mm Nova-Pak ® C18 4 µm reverse phase column (Waters Corporation, Milford, MA).

Samples of timothy hay cubes, alfalfa hay cubes, and oats were collected during period 1, period 5, period 6 and period 10 and sent to a commercial laboratory for proximate analysis via wet chemistry (Dairy One Cooperative Inc., Ithaca, NY). Feed amino acid concentrations were determined using acid hydrolysis (AOAC International, 2005). Samples were ground, and 0.2 g was weighed into ashed vials. To these vials, 12 mL of 6N hydrochloric acid (Fisher Scientific, Fair Lawn, NJ) was added and samples were capped tightly and incubated at 110 °C for 24 h. After incubation, samples were filtered using a 0.45 µm syringe filter (Sarstedt, Numbrecht, Germany) into microcentrifuge tubes. Samples were then derivatized and analyzed by HPLC, as described for plasma samples. To measure methionine concentrations, a separate assay was performed in which 2 mL of performic acid (J.T. Baker Chemicals, Center Valley, PA) and 0.42 g of metabisulfite (Fisher Scientific, Fair Lawn, NJ) were added to samples and incubated overnight at 4 °C prior to the addition of hydrochloric acid (AOAC International, 2005).

The isotopic enrichment of plasma samples collected during isotope infusion was determined by a commercial laboratory using negative chemical ionization GC-MS analysis of a heptafluorobutyric, n33 propyl derivative (Metabolic Solutions Inc., Nashua, NH), as previously described (Matthews et al., 1990; Wagner, 2013). A Phenomenex ZB-1MS capillary column was used to separate the derivative of phenylalanine. Selected ion chromatograms were

obtained by monitoring ions at a m/z 383 and 384 for phenylalanine and [1-^{13}C]phenylalanine, respectively. The isotope enrichment of breath samples was determined by measuring the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the breath using an infrared isotope analyzer (IRIS-3; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany) (Urschel et al., 2012).

Calculations

The average enrichment at isotopic stable state was determined for both plasma and breath samples. The plateau was defined as at least 3 points with a coefficient of variation less than 10%. If the plateau was not obtained, the data was discarded. Total CO_2 production rate was determined using the average enrichment of breath samples during [^{13}C] sodium bicarbonate infusion with the following formula:

$$CO_2 \text{ production} = i \times [Ei/Eb) - 1] \times [0.0224\, ml/\mu mol\ CO_2]$$

where *i* is rate of the isotope infusion (μmol/(kg BW·h)), *Ei* is the enrichment of isotope solution, and *Eb* is the plateau breath enrichment (Hoerr et al., 1989). Whole-body phenylalanine flux is the rate of phenylalanine entering into the free amino acid pool, which is also equivalent to rate of exit of phenylalanine from the free amino acid pool. Flux was calculated using the following equation where Q is flux (μmol/(kg BW·h)), *i* is rate of the isotope infusion (μmol/(kg BW·h)), *Ei* is isotope solution enrichment, and *Ep* is the plateau plasma enrichment (Hsu et al., 2006):

$$Q = i \times [(Ei/Ep) - 1]$$

The different processes affecting flux include amino acids entering the blood amino acid pool from dietary intake (I), *de novo* synthesis (N), and protein breakdown (B), or leaving the pool through protein synthesis (Z), oxidation (E), or the conversion to other metabolites (M) (Picou and Taylor-Roberts, 1969):

$$Q = I + N + B = Z + E + M$$

Phenylalanine entering the plasma amino acid pool from dietary intake (I) was corrected for pre-cecal digestibility by multiplying phenylalanine intake from forage by factor of 0.4 (Gibbs et al., 1988) and multiplying the phenylalanine intake from concentrate by a factor of 0.7 (Farley et al., 1995), and further corrected by assuming that 26.5% of the digestible phenylalanine was extracted by the splanchnic tissues (Mastellar et al., 2016a). Because mammals are not able to synthesize phenylalanine *de novo*, the phenylalanine entering the blood amino acid pool from protein breakdown (B) can be determined using the following equation:

$$B = Q - I$$

[1-^{13}C] phenylalanine oxidation was calculated using the following equation (Hsu et al., 2006):

$$E = F^{13}CO_2\ (1/Ep - 1/Ei) \times 100$$

where E represents phenylalanine oxidation (μmol/ (kg BW·h)) and $F^{13}CO_2$ is the product of isotope enrichment of the breath and the rate of carbon dioxide production (μmol/ (kg BW·h)). Tyrosine was believed to be provided in excess for all treatments, therefore the conversion of phenylalanine to tyrosine (M) was

considered negligible. Therefore, protein synthesis can be estimated using the following equation:

$$Z = Q - E$$

Statistical Analysis

All experimental data were analyzed using the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC), with significance set at $P \leq 0.05$. Measurements are presented as least squares means ± standard error of the mean, or as least squares means with a pooled standard error of the mean where applicable.

Plasma urea nitrogen and plasma amino acid concentrations were analyzed using repeated measures, with time, treatment and their interaction as the fixed effects and horse as the random subject. A compound symmetry variance-covariance structure was chosen based on lowest values for respective fit statistics (AIC).

To further analyze treatment differences within both the pre-feeding and 90 min post-feeding time points, treatments were separated into two variables: forage type and amino acid supplement. Plasma urea nitrogen and plasma amino acid concentrations at each timepoint were analyzed as a two-way ANOVA with forage, amino acid supplement and their interaction as the fixed effects and horse was included as a random effect.

A one-way ANOVA was used to analyze treatment effects on whole-body phenylalanine kinetics with treatment as the fixed effect, and horse as the random effect. To better characterize the effect of forage type and amino acid supplement on whole-body phenylalanine kinetics, this data was also analyzed as a 2x2 factorial design, with forage, amino acid supplement and their interaction as the fixed effects and horse was included as a random effect.

For all cases, when fixed effects were significant, means were separated using the Tukey-adjusted pdiff option.

Results

All horses completed all of the experimental treatments and sampling procedures. Feed refusals were uncommon, particularly on sampling days, but when orts were left they were documented and accounted for. Additionally, all horses consumed all of the experimental diets during all adaptation periods. All horses remained healthy for the duration of the study.

Plasma urea nitrogen concentrations

When analyzing plasma urea nitrogen concentrations for overall treatment and time effects, there were significant differences seen between treatments ($P = 0.002$) and differences between pre and post-feeding blood samples ($P = 0.02$), however the interaction was not significant ($P > 0.05$) (Table 4.3A and Table 4.3B). Plasma urea nitrogen concentrations were greatest on average when horses consumed the alfalfa treatments, compared to the timothy treatments. Generally,

90-minutes post feeding concentrations were greater than or equivalent to pre-feeding values of plasma urea nitrogen concentrations. To better characterize the effects of forage and amino acid supplement on plasma urea nitrogen concentrations, the data at both time points was then analyzed as a 2x2 factorial design. For the pre-feeding samples, there was a significant effect of forage type on plasma urea nitrogen concentration ($P < 0.0001$) (Table 4.4), with the alfalfa treatments resulting in greater plasma concentrations than the timothy treatments. There was no significant effect ($P > 0.05$) of either supplemented amino acid or the interaction between forage and supplemented amino acid on pre-feeding plasma urea nitrogen concentrations. When the post-feeding samples were analyzed, there was a significant effect of forage type on plasma urea nitrogen concentration ($P < 0.0001$), with the alfalfa treatments resulting in higher concentrations than the timothy treatments (Table 4.5). There was no significant effect of amino acid supplement or of the forage by amino acid supplement interaction on post-feeding plasma urea nitrogen concentrations.

Plasma indispensable amino acid concentrations

Treatment significantly affected the plasma concentrations of histidine ($P < 0.0001$), leucine ($P = 0.02$), lysine ($P < 0.0001$), methionine ($P = 0.02$) and threonine ($P < 0.0001$; Table 4.3A and Table 4.3B). When diets were supplemented with histidine, lysine, threonine, or + (Con), plasma concentrations of those amino acids were greater compared to the treatments not supplemented with each of those amino acids. Plasma concentrations of leucine were greatest

when horses consumed the alfalfa cube treatments compared to when horses consumed the timothy cube treatments. Methionine plasma concentrations were greatest when horses consumed the timothy cube treatments compared to when horses consumed alfalfa cube treatments. There was a significant effect of time on plasma histidine ($P < 0.0001$), lysine ($P < 0.0001$), methionine ($P = 0.003$) and threonine ($P < 0.0001$) concentrations, where the 90 min post-feeding concentrations were greater than the pre-feeding concentrations (Table 4.3A and Table 4.3B). Plasma concentrations of lysine, threonine and histidine were greatest in horses consuming those amino acids as dietary supplements and there was a more pronounced feeding effect. There was a significant interaction of treatment by time for lysine ($P < 0.0001$), threonine ($P < 0.0001$), histidine ($P < 0.0001$) and methionine ($P = 0.003$) plasma amino acid concentrations ($P < 0.0001$; Table 4.3A and Table 4.3B). When horses consumed lysine, threonine, histidine, or the (+) Con diet with all three supplements, the respective plasma amino acid concentrations rose in response to feeding. When horses consumed diets supplemented with either threonine or the (+) Con diet, plasma methionine concentrations were greater, regardless of forage type.

To better understand the treatment effects, data were separated by time point and then analyzed as a 2x2 factorial design. Pre-feeding plasma methionine concentrations were affected by forage ($P = 0.05$), with timothy resulting in higher plasma concentrations than the alfalfa (Table 4.4). Pre-feeding plasma threonine concentrations were affected by amino acid supplement ($P = 0.002$), with the two supplements containing additional threonine (threonine and (+) Con)

having the highest pre-feeding concentrations (Table 4.4). However, when horses consumed the alfalfa and (+) Con diet, pre-feeding plasma concentrations of threonine were not different from when horses consumed the other supplements, except for when they consumed alfalfa and (-) Con treatments. When horses consumed alfalfa and (-) Con treatments, plasma threonine concentrations were significantly lower than when horses consumed alfalfa and (+) Con and alfalfa and threonine. There was no other significant forage, amino acid supplement or forage by amino acid supplement interaction effects on any of the other pre-feeding indispensable plasma amino acid concentrations ($P < 0.05$; Table 4.4). In the post-feeding samples, there was a significant effect of forage type on plasma isoleucine ($P < 0.0001$), leucine ($P < 0.0001$), phenylalanine ($P = 0.04$) and valine ($P = 0.0004$) concentrations (Table 4.5). Overall, when horses consumed the alfalfa-based diets concentrations of plasma isoleucine, leucine, phenylalanine and valine were greater than when horses consumed the timothy-based diets. Post-feeding, there was a significant effect of amino acid supplementation on plasma histidine ($P < 0.0001$), lysine ($P < 0.0001$), threonine ($P < 0.0001$) and tryptophan ($P = 0.01$) concentrations (Table 4.5). For the histidine, lysine and threonine concentrations, plasma concentrations were greatest when horses consumed the treatments supplemented with the respective amino acids. Plasma tryptophan concentrations were greatest when horses consumed diets supplemented with threonine, but were not different compared to when horses consumed diets supplemented with histidine. There were no significant effects of

the forage type by amino acid supplement interaction for any of the post-feeding plasma indispensable amino acid concentrations ($P > 0.05$; Table 4.5).

Plasma dispensable amino acid concentrations

There were no significant differences in plasma dispensable amino acid concentrations in response to treatment ($P > 0.05$; Table 4.3A and Table 4.3B). Dispensable plasma amino acid concentrations increased in the post-feeding samples when compared to the pre-feeding samples ($P < 0.05$), except for aspartate, glutamate, glycine and tyrosine, where there was no effect of time ($P > 0.05$; Table 4.3A and Table 4.3B). There was a significant interaction of treatment by time for plasma alanine, aspartate, glutamate, glycine, and serine ($P < 0.05$) concentrations (Table 4.3A and Table 4.3B). There was a significant increase in plasma alanine concentrations following feedings of the alfalfa and histidine, alfalfa and (+) Con, timothy and threonine, timothy and histidine and timothy, and (+) Con treatments. For all other treatments, there was no effect of time on plasma alanine concentrations. Following the feeding of alfalfa and threonine, alfalfa and (+) Con, and timothy and (-) Con, there was a significant increase in plasma aspartate concentrations. There was no effect of time on plasma aspartate concentrations for any of the other treatments. There was a significant increase in plasma glutamate concentrations following the feedings of alfalfa and (-) Con, alfalfa and (+) Con, and timothy and (-) Con. For all other treatments, there was no effect of time on plasma glutamate concentrations. Following the feeding of alfalfa and (-) Con, alfalfa and threonine and timothy and threonine, there was a

significant increase in plasma glycine concentrations. There was no effect of time on plasma glycine concentrations for any of the other treatments. There was a significant increase in plasma serine concentrations following feedings of alfalfa and threonine, histidine and (+) Con, and timothy and threonine, histidine and (+) Con. For all other treatments, there was no effect of time on plasma serine concentrations.

To better characterize the treatment effects, data were analyzed as a 2x2 factorial design. In pre-feeding plasma samples, none of the dispensable amino acid concentrations were affected by forage type, amino acid supplementation or the interaction of forage type by amino acid supplement ($P > 0.05$; Table 4.4). In post-feeding plasma samples, there was a significant effect of forage type on plasma alanine and aspartate concentrations ($P < 0.05$), where concentrations were greater when horses consumed timothy-based diets compared to alfalfa-based diets (Table 4.5). There was a significant effect of amino acid supplementation on post-feeding plasma alanine, asparagine, aspartate, citrulline, glutamate, glutamine glycine, proline, serine and tyrosine concentrations ($P < 0.05$), as shown in Table 4.5. There were no significant interactions of forage type by amino acid supplement seen for any of the post-feeding, dispensable amino acid plasma concentrationsa ($P > 0.05$; Table 4.5).

Whole-body phenylalanine kinetics

There was a significant effect of treatment on phenylalanine flux ($P < 0.0001$; Table 4.6). When horses consumed the alfalfa-based diets, phenylalanine

flux was greater than when horses consumed the timothy diets. There was also a significant treatment effect on phenylalanine oxidation (*P = 0.00014*; Table 4.6). The alfalfa and threonine treatment had the greatest rates of phenylalanine oxidation, whereas the timothy and (-) Con treatment had the lowest, with all other treatments being intermediate. The difference between phenylalanine flux and oxidation represents non-oxidative phenylalanine disposal, which is an estimate of phenylalanine use for protein synthesis. There was an effect of treatment on phenylalanine use for protein synthesis (*P = 0.05*; Table 4.6). The alfalfa and lysine diet had the greatest phenylalanine use for protein synthesis, whereas the timothy and histidine diet had the lowest estimated use for protein synthesis, and all remaining treatments were intermediate. There was a significant difference between all the alfalfa and timothy treatments with respect to phenylalanine intake (*P < 0.0001*; Table 4.6). There was a greater intake of phenylalanine in the five alfalfa treatment groups when compared to the five timothy treatment groups. There was no significant effect of treatment on phenylalanine release from protein breakdown or total CO_2 production (*P > 0.05*; Table 4.6).

In order to discern which treatment factors were responsible for the significant differences across treatments, data were analyzed with forage type and amino acid supplement as two variables in a factorial design. There was an effect of forage (*P = 0.0025*) and the interaction of forage by amino acid supplement (*P = 0.04*), but no effect of amino acid supplement, on phenylalanine flux (*P = 0.04*; Table 4.7). Overall, the alfalfa treatments resulted in a greater phenylalanine flux

when compared to the timothy treatments. When horses received alfalfa supplemented with either lysine or histidine, phenylalanine flux was greater compared to when horses consumed timothy diets supplemented with the same amino acids, whereas for all other amino acid supplements, there was no effect of forage type of phenylalanine flux. Phenylalanine oxidation was significantly affected by forage type ($P = 0.0008$) but not by amino acid supplement or the forage type by amino acid supplement interaction ($P > 0.05$; Table 4.7). Higher rates of phenylalanine oxidation were seen with the alfalfa forage when compared to the timothy forage. There was a significant interaction of forage by amino acid supplement, with respect to phenylalanine use for protein synthesis ($P = 0.05$); however, no significant effect of either forage type or amino acid supplement ($P > 0.05$; Table 4.7). Within the alfalfa forage type, phenylalanine use for protein synthesis was greatest when horses consumed lysine and lowest when horses consumed threonine, but there was no effect of amino acid supplement when horses received the timothy treatment (Table 4.7). Phenylalanine release from protein breakdown followed a similar pattern, with a significant interaction between forage type and amino acid supplement ($P = 0.05$), but there was no effect of forage type or amino acid supplement ($P > 0.05$; Table 4.7). Similarly, phenylalanine release from protein degradation in the horses receiving the alfalfa forage was greatest when horses consumed lysine and lowest when horses consumed threonine, but there was no effect of amino acid supplement when horses received the timothy forage (Table 4.7). Dietary phenylalanine intake was significantly affected by forage type ($P < 0.0001$), where horses consuming

alfalfa received more phenylalanine than horses consuming timothy. Phenylalanine intake was, however, not affected by either amino acid supplement or the forage type by amino acid supplement interaction ($P > 0.05$; Table 4.7). There was no effect of forage type, amino acid supplement or their interaction on CO_2 production ($P > 0.05$; Table 4.7).

Discussion

This study used isotopic techniques and the measurement of blood metabolites to study whole-body protein metabolism in horses fed either alfalfa or timothy hay cubes, and a small meal of oats, that had been fortified with amino acids that have previously been suggested to be potentially limiting in horses. In general, there was no effect of amino acid supplementation observed for either forage treatment, indicating that these amino acids were unlikely to be limiting whole-body protein synthesis. Although the alfalfa forage provided more of the indispensable amino acids than the timothy forage, it did not appear that any of the amino acids studied were limiting protein synthesis in either forage. With respect to whole-body protein metabolism, the results of this study indicate that mature horses at maintenance derived no additional benefit from consuming a primarily alfalfa diet compared to consuming timothy-based diets. The effect of forage species was the most influential aspect of the observed results, which was expected considering forage was the major component of the treatment diets.

All diets were formulated to meet and slightly exceed energy requirements so that protein synthesis would not be limited by energy intake (NRC, 2007;

Table 4.1). Horses' bodyweight (BW) did not change in the duration of the study, indicating that the maintenance ration was fed appropriately. It is estimated that horses can willingly eat 2-3% of their BW each day as forage (NRC, 2007). Horses in this study were offered forage at 2.0% of BW, on an as fed basis, and 1kg of oats per day, which was split into two meals and feed refusals were sparse. This feeding regime is classified as a high-forage diet as it provided approximately 92% of the horses' diets as forage with the remaining 8% was provided as oats. When horses were fed the alfalfa-based treatments, they consumed 3.01 g/kg BW/d of CP, which is well above the NRC's CP requirement of 1.26 g/kg BW/d for a 573 kg mature horse at maintenance (NRC, 2007;). When horses were fed the timothy-based treatments, they consumed 2.21 g/kg BW/d of CP, which was also above the NRC's requirement (NRC, 2007).

It has been reported in humans that amino acid flux and oxidation both increase as protein content of the diet increases (Motil et al., 1981). Non-oxidative phenylalanine disposal, which is the difference between phenylalanine flux and oxidation, is an estimate of phenylalanine use for protein synthesis. Based on these calculations, we can use these parameters as a tool to estimate the fate of amino acid use and measure changes in whole-body protein metabolism. Isotope kinetic methodologies are able to detect differences in whole-body protein metabolism with adaptation times of less than a week and have been extensively used in studies in swine and humans (Zello et al., 1990; Pencharz and Ball, 2003; Myrie et al., 2008; Elango et al., 2009; Myrie et al., 2014) and more recently in horses (Mastellar et al., 2016a; Mastellar et al., 2016b; Mok et al., 2018; Urschel

et al., 2012; Wagner et al., 2013). Factors such as the subject's age (Volpi et al., 2001), diet (Tanner et al., 2014; Zello et al., 1993) or the timing of post-exercise nutrient intake (Okamura et al., 1997) all have the capability to influence amino acid kinetics. Whole-body movement of amino acids can be modeled by stable isotope infusion of an amino acid, in this case phenylalanine, into and out of the free amino acid pool. Estimates of rates of phenylalanine oxidation, use for protein synthesis and release from protein degradation can also be made using these methodologies. It has previously been shown that rates of protein synthesis are equal to rates of protein breakdown in mature horses not accreting protein, indicating that individual indispensable amino acid requirements were met by the diet (Urschel et al., 2012). Despite differences with forage treatment, little to no effect of amino acid supplementation was seen on any of the kinetics parameters studied. This, combined with the plasma urea nitrogen results, indicates that none of the amino acids studied were limiting in the diets formulated for this study. This leads to the conclusion that mature horses receiving adequate amounts of a good quality forage, regardless of whether it is timothy or alfalfa, can maintain similar rates of whole-body protein synthesis without the need for supplemental lysine, threonine or histidine.

The lack of a forage effect on phenylalanine use for protein synthesis, coupled with the presence of an effect of forage on phenylalanine flux and oxidation could be explained by greater phenylalanine intake in the alfalfa-based diets, resulting in higher flux, and higher oxidation within those horses. This has been previously reported in mature horses consuming a complete senior feed

supplemented with either lysine, leucine, or phenylalanine, using a glutamate supplement as a control (Urschel et al., 2012). When horses consumed the phenylalanine supplement, phenylalanine flux and oxidation were greater than in those fed the control diet (Urschel et al., 2012). However, there was no effect of dietary treatment seen on measures of either non-oxidative phenylalanine disposal or phenylalanine release from protein breakdown (Urschel et al., 2012). None of the supplemental amino acids supported an increase in the rate of whole-body protein synthesis compared to the control diet, which agrees with findings from the present study (Urschel et al., 2012). These findings support that none of the supplemented amino acids were limiting to protein synthesis.

It is recommended that sedentary mature horses receive 54 mg/kg BW/d of lysine, which was exceeded by both alfalfa (146 mg/kg BW/d) and timothy-based diets (120 mg/kg BW/d) (NRC, 2007), even without additional lysine supplementation. Recommendations for other indispensable amino acids can be made based on the assumption that the amino acid composition of muscle tissue is representative of whole-body protein composition in growing horses (Bryden, 1991). Therefore, the ratio of indispensable amino acids to lysine in muscle tissue are used to estimate recommendations for dietary indispensable amino acids. All calculatable recommendations for indispensable amino acids were exceeded by both dietary treatments (NRC, 2007). Excess amino acids cannot be stored in the body above the limiting most amino acids, or above the rate of protein synthesis, therefore they are marked for catabolism. When amino acids are catabolized endogenously, the amino moiety is eventually converted to urea via the hepatic

urea cycle and the carbon backbones are converted to CO_2 and disposed of via exhalation. For this reason, urea nitrogen concentrations are often used as a measure of amino acid degradation.

Urea nitrogen concentrations from serum, plasma and whole blood have been used to predict nitrogen excretion and efficiency of nitrogen utilization in many different species, including horses (Graham et al., 1994; Martin et al., 1996; Kohn et al., 2005; Mantovani et al., 2014). The average range of reference of serum urea nitrogen concentrations in mature horses is approximately 3.5 – 8 mmol/L, which is comparable to range of values seen in horses in this study (4.7 – 6.3 mmol/L) (Cornell University Veterinary Diagnostics Lab, 2018). When horses consumed alfalfa-based diets plasma urea nitrogen concentrations were greater than when they consumed timothy-based diets, both pre and post feeding, which supports that more urea was formed when horses consumed alfalfa when compared to timothy. This suggests that either more amino acids were broken down when the alfalfa forage was fed, or alternatively that there was less amino acid breakdown when consuming the timothy diet. Because amino acids cannot be stored beyond what is needed to support protein synthesis, excess amino acids in need of catabolism results in elevated plasma urea nitrogen concentrations.

Higher intake of amino acids and increased plasma urea nitrogen concentrations within the alfalfa-based treatments suggest these additional amino acids did not appear to be efficiently incorporated into body protein. In another study, significantly greater urinary nitrogen losses were observed in mature

horses consuming a high protein (24.9% CP) alfalfa hay when compared to horses receiving a low protein (18.8% CP) alfalfa hay (Woodward et al., 2011). However, while nitrogen intake and fecal nitrogen numerically increased in that study, they not differ significantly between the different protein treatments (Woodward et al., 2011). Although urinary nitrogen output was not measured in the present study, this data supports that the additional amino acids provided over the horse's requirements by the alfalfa-based dietary treatments in the present study were likely metabolized and marked for excretion. Despite the overall effect of forage type on plasma urea nitrogen concentrations, there was no further effect of any of the supplemental amino acids on plasma urea nitrogen concentrations. Additionally, supplementation did not increase amino acid catabolism then, because the addition of amino acids to the diet did not increase plasma urea nitrogen concentrations. This indicates that the addition of the various supplemental amino acids did not improve dietary protein utilization. This provides evidence that the amino acids supplied by the forage were sufficient to support protein synthesis and none were limiting.

When horses consumed the lysine, threonine or histidine supplements, plasma amino acid concentrations of those amino acids rose in response to feeding. It has previously been shown in horses that plasma concentrations of amino acids, such as lysine and threonine, are responsive to dietary inclusion levels (Graham-Thiers and Bowen, 2011; Mastellar et al., 2016a; Mastellar et al., 2016b; Mok et al., 2018; Ohta et al., 2007). Plasma concentrations of indispensable amino acids are able to be influenced by whole-body protein

turnover, rate of amino acid degradation and dietary intake (Urschel et al., 2012; Tanner et al., 2014; Mastellar et al., 2016a). Therefore, it was expected that plasma amino acid concentrations for histidine, lysine, threonine would be significantly increased after consumption of the histidine, lysine, threonine and (+) Con treatments, which was confirmed by the results of this study. There was a difference in plasma glutamate concentrations when horses consumed the (-) Con treatment. This provides evidence that the treatment allocations were effective and that they were at least partially digested and absorbed after 90 minutes, which is consistent with findings from other studies in horses (Essen-Gustavsson et al., 2010; Mok et al., 2018).

In the postprandial state, there is an increase in whole-body amino acid metabolism as some amino acids are incorporated into protein while others are used to make dispensable amino acids or other metabolites. Additionally, any excess amino acid will be catabolized through a variety of degradation pathways, as reviewed by Brosnan (2003). Consequently, there is a huge shift in inter-organ amino acid fluxes compared to the post-absorptive state. As tissue amino acid metabolism increases, important nitrogen carriers responsible for transamination reactions, such as glutamine and alanine, are released into circulation, in order to maintain whole-body energy and protein homeostasis. After feeding, concentrations of alanine and glutamine increased significantly when horses' diets were supplemented with threonine or the (+) Con diet including threonine compared to forages supplemented with lysine or the (-) Con treatment. Alanine and glutamine are known nitrogen carriers that readily donate and receive amino

groups, through different transamination reactions, particularly by accepting nitrogen from branched-chain amino acid breakdown (Brosnan, 2003). Plasma alanine and glutamine levels are typically elevated in post-absorptive and catabolic states due to their importance in immune function, glucose homeostasis, and interorgan nitrogen balance. Therefore, the body's increased metabolism of glutamine and alanine, and associated catabolism of BCAA, play a major role in amino acid metabolism under catabolic conditions (Holeček, 2002). Transamination reactions help to clear blood levels of excess ammonia in times of amino acid excess, and also provide transportation of amine groups and carbon backbones to other tissues for *de novo* protein synthesis in the body (Christensen, 1990). Although amino acids were provided at adequate levels according to the NRC's recommendations (NRC, 2007), it is possible that horses consuming timothy-based diets required more mobilization of nitrogen carriers to plasma in order to maintain homeostatic functions.

Interestingly, pre-feeding plasma methionine concentrations were numerically greater when horses consumed the timothy-based diets when compared to the alfalfa-based diets, despite the timothy cubes having lower methionine concentration than the alfalfa cubes. It is currently unclear why this this occurred, however increased levels of any amino acid may indicate that they were possibly not taken up by tissues or that there was an increased release from tissue. Because circulating blood amino acid levels are a function of tissue uptake and release, it could be speculated that less methionine was taken up or that more was released when horses consumed the timothy-based diets. Methionine

metabolism has been reviewed by Finkelstein (1990) and has been found to be essential for proper growth and homeostasis due to its roles in numerous biological processes such as protein synthesis, transmethylation reactions, polyamine formation, cystathionine and cysteine synthesis. How methionine metabolism was affected by the timothy treatment in this study and what effects this may have had endogenously are areas warranting further investigation.

A limitation of this study was that the digestibility of the hay cubes used was not determined; however, it is important to note that differences with respect to forage type and level of CP have been reported. Another study illustrated that total tract protein digestibility in mature geldings was found to be closely related to the protein content of the forage, where legumes were found to have significantly more digestible crude protein (Fonnesbeck et al., 1967). The geldings reportedly consumed greater amounts of legume than grass forage, signifying an intake preference (Fonnesbeck et al., 1967). Growing horses have been reported to preferentially consume more alfalfa than grass hays and that the apparent digestibility of organic matter was significantly greater when horses consumed alfalfa than when they consumed grasses (LaCasha et al., 1999). Conversely, other studies showed that apparent total tract digestibility appeared to be not largely different between these forage types, where alfalfa hay with a 15% CP was 66% digestible in ponies, and that of timothy hay with a 9.9% CP was 65% in mature horses (Gibbs et al., 1988; Darlington et al., 1968). Together, these data suggest that there may be a correlation between preferential intake and apparent total tract digestibility in horses and that protein from legume hay might

be more digestible than that of grass hays. Although we did not measure the digestibility of our diets, this would explain some of the results seen in the current study. In general phenylalanine flux, oxidation, and use for protein synthesis was higher when horses consumed the alfalfa-based diets when compared to the timothy treatments. This could be a consequence of higher protein intake or greater digestibility within the alfalfa-based diets.

Another limitation of this study is that by only using two levels of amino acid intake, it is not possible to determine from the indispensable plasma amino acid concentrations alone if one of the amino acids supplemented may have been limiting protein synthesis. However, it is known that as amino acid intake increases above the limit of protein synthesis, an increase in plasma concentrations occurs with increasing intake (Munro, 1978). Based on the comparison of recommendations for indispensable amino acids to average daily nutrient intakes (Table 4.2), it appears that the un-supplemented diets met the requirements for all dietary indispensable amino acids. Although plasma amino acid concentrations often reflect changes in whole-body protein metabolism, this measure provides only a segment of the whole-body free amino acid pools and does not allow for definitive conclusions regarding the underlying mechanisms influencing the changes that occur. Samples were collected for only two timepoints, pre- and post-feeding, so this limits the ability to draw further conclusions regarding amino acid pool fluctuations over time. Another limitation of using changes in plasma amino acid concentration as a criterion for amino acid requirements is that it only accounts for free amino acids, and not amino acids

bound in the tissues or in other proteins found in other plasma components, such as glycoproteins, lipoproteins or albumin (Krebs, 1950).

Conclusion

The results of this study indicate that mature horses at maintenance received no benefit with respect to whole-body protein metabolism from consuming the primarily alfalfa diet, when compared to consuming the timothy-based diets. If mature horses at maintenance can fulfill protein requirements from a quality forage-based diet, additional supplementation from concentrates or free amino acids are catabolized, and thus wasted, resulting in elevated plasma urea nitrogen concentrations. There was no effect of forage on phenylalanine use for protein synthesis, however there was an effect of forage on phenylalanine flux and oxidation. This is likely related to the fact that phenylalanine intake was greater in the alfalfa group, resulting in higher flux, and higher oxidation to remove the excess phenylalanine from the body. Measures of whole-body protein synthesis indicate that alfalfa-based diets supported similar rates of protein synthesis when compared to horses fed timothy-based diets, and there was no apparent benefit to any of the supplemental amino acids in promoting elevated rates of whole-body protein synthesis. There was no evidence to support that either histidine, lysine or threonine were limiting to whole-body protein metabolism in mature horses at maintenance, consuming either a timothy or alfalfa cube-based diet in this study.

Tables

Table 4.1: Nutrient composition of each component of treatment diet, as-fed basis.

	Oats	Alfalfa Cubes	Timothy Cubes
Overall nutrient composition[1]			
Dry Matter, %	87.63 ± 0.32	89.83 ± 0.49	89.78 ± 0.38
DE, Mcal/kg	3.31 ± 0.04	1.75 ± 0.04	1.81 ± 0.01
Crude protein, %	10.83 ± 0.51	14.08 ± 0.79	10.08 ± 0.70
Estimated lysine, %	0.45 ± 0.03	0.72 ± 0.04	0.35 ± 0.02
Lignin, %	2.15 ± 0.52	8.53 ± 0.50	5.50 ± 0.32
Acid Detergent Fiber, %	6.60 ± 0.62	38.78 ± 1.33	35.78 ± 1.68
Neutral Detergent Fiber, %	11.23 ± 1.48	50.55 ± 1.55	51.90 ± 0.48
Water Soluble Carbohydrates, %	3.13 ± 0.28	5.18 ± 0.71	11.43 ± 0.77
Ethanol Soluble Carbohydrates, %	2.38 ± 0.53	3.18 ± 0.75	6.78 ± 0.94
Starch, %	52.35 ± 0.90	0.75 ± 0.47	0.60 ± 0.32
Non-fibrous carbohydrates, %	58.28 ± 1.78	13.63 ± 1.31	17 ± 0.95
Crude fat, %	5.38 ± 0.17	1.5 ± 0.08	1.65 ± 0.17
Ash, %	1.93 ± 0.19	10.08 ± 0.40	9.15 ± 0.39
Calcium, %	0.08 ± 0.01	1.03 ± 0.20	0.56 ± 0.03
Phosphorus, %	0.29 ± 0.02	0.18 ± 0.02	0.2 ± 0.02
Magnesium, %	0.1 ± 0.01	0.25 ± 0.01	0.28 ± 0.04
Potassium, %	0.39 ± 0.03	2.03 ± 0.15	1.53 ± 0.11
Sodium, %	0.01 ± 0.01	0.04 ± 0.01	0.08 ± 0.03
Chloride, %	0.09 ± 0.01	0.20 ± 0.03	0.26 ± 0.02
Sulfur, %	0.15 ± 0.01	0.20 ± 0.01	0.18 ± 0.01
Iron, mg/kg	55 ± 5.38	720 ± 192.08	880 ± 261.92
Zinc, mg/kg	35 ± 1.89	25 ± 1.50	85 ± 18.37
Copper, mg/kg	6.50 ± 1.00	9.00 ± 1.41	49 ± 24.23
Manganese, mg/kg	47 ± 2.99	46 ± 3.59	102 ± 23.11
Molybdenum, mg/kg	0.78 ± 0.05	1.30 ± 0.14	0.68 ± 0.17
Cobalt, mg/kg	0.20 ± 0.05	0.43 ± 0.20	0.64 ± 0.18
Amino acid composition, % [2]			
Alanine	0.41 ± 0.03	0.52 ± 0.05	0.47 ± 0.04
Arginine	0.67 ± 0.05	0.49 ± 0.05	0.41 ± 0.04
Aspartate and Asparagine	0.77 ± 0.06	1.01 ± 0.10	0.74 ± 0.08
Glutamate and Glutamine	1.98 ± 0.15	1.04 ± 0.09	0.88 ± 0.07
Glycine	0.41 ± 0.03	0.45 ± 0.04	0.37 ± 0.03
Histidine	0.21 ± 0.01	0.19 ± 0.02	0.15 ± 0.02
Isoleucine	0.36 ± 0.03	0.44 ± 0.06	0.34 ± 0.06
Leucine	0.70 ± 0.05	0.74 ± 0.08	0.62 ± 0.05
Lysine	0.36 ± 0.02	0.46 ± 0.05	0.32 ± 0.04
Methionine	0.41 ± 0.05	0.28 ± 0.16	0.06 ± 0.02
Phenylalanine	0.50 ± 0.04	0.49 ± 0.05	0.39 ± 0.04
Proline	0.48 ± 0.03	0.69 ± 0.06	0.56 ± 0.14
Serine	0.45 ± 0.03	0.48 ± 0.07	0.33 ± 0.06
Threonine	0.31 ± 0.02	0.35 ± 0.03	0.29 ± 0.05
Tyrosine	0.31 ± 0.03	0.29 ± 0.04	0.23 ± 0.02
Valine	0.49 ± 0.04	0.56 ± 0.07	0.45 ± 0.07

[1]Analyzed by Equi-Analytical Laboratories

[2]Analyzed by HPLC in our lab

Table 4.2: Calculated average daily nutrient intakes of mature horses fed either alfalfa or timothy-based diets

	Feedstuff		NRC (2007) Recommendation[1]
Nutrient	Alfalfa + Oats	Timothy + Oats	Total Daily Intake
Digestible energy, Mcal/d	0.041	0.042	0.030
Crude protein, g/kg BW/d	3.01	2.21	1.26
Indispensable amino acids, mg/kg BW/d			
Alanine	215	161	
Arginine	243	211	41
Aspartate and Asparagine	104	74	
Glutamate and Glutamine	97	81	
Glycine	42	34	
Histidine	110	94	31
Isoleucine	75	63	30
Leucine	111	101	58
Lysine	146	120	54
Methionine	63	51	15
Phenylalanine	121	99	32
Proline	63	19	
Serine	94	74	
Threonine	160	136	33
Tyrosine	107	87	
Valine	98	70	33

[1] Calculated based on the recommendation for sedentary mature horse weighing 573 kg as determined by the NRC.

Results presented on an as-fed basis.

Forages were provided at 2% of bodyweight on an as-fed basis.

Oats were provided at 1kg per day on an as-fed basis.

Table 4.3A: Main effects of treatment and time on plasma concentrations of urea nitrogen and amino acids in mature horses fed diets with either alfalfa or timothy combined with amino acid supplements.

	Treatment										P-values		
	Alfalfa												
Metabolite	**(-) Control**		**Lysine**		**Threonine**		**Histidine**		**(+) Control**		**Treatment**	**Time**	**Treatment * Time**
Sample Time	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**			
PUN, mmol/L	6.3 ± 0.3^{A}	6.2 ± 0.3^{a}	5.6 ± 0.3^{BCD}	5.8 ± 0.3^{abc}	6.1 ± 0.3^{AB}	6.2 ± 0.3^{a}	5.9 ± 0.3^{ABC}	6.0 ± 0.3^{ab}	5.9 ± 0.3^{ABC}	6.0 ± 0.3^{ab}	0.002	0.02	0.92
Indispensable Amino Acids, µmol/L													
Histidine	67 ± 18^{BC}	72 ± 18^{c}	79 ± 17^{ABC}	105 ± 17^{c}	61 ± 18^{C}	79 ± 18^{c}	80 ± 16^{BC}	182 ± 16^{b*}	84 ± 16^{AB}	246 ± 17^{a*}	<0.0001	<0.0001	<0.0001
Isoleucine	87 ± 6^{AB}	81 ± 6^{ab}	92 ± 6^{A}	85 ± 6^{ab}	81 ± 6^{ABC}	84 ± 6^{ab}	85 ± 6^{ABC}	88 ± 6^{a}	87 ± 6^{AB}	85 ± 6^{ab}	0.07	0.4	0.92
Leucine	156 ± 11^{AB}	143 ± 11^{ab}	167 ± 10^{A}	152 ± 10^{a}	160 ± 11^{AB}	155 ± 11^{a}	154 ± 10^{AB}	156 ± 10^{a}	162 ± 10^{A}	151 ± 10^{ab}	0.02	0.06	0.94
Lysine	94 ± 25^{AB}	87 ± 25^{b}	100 ± 24^{AB}	288 ± 26^{a*}	85 ± 25^{B}	94 ± 25^{b}	95 ± 24^{AB}	138 ± 22^{b}	102 ± 22^{AB}	320 ± 24^{a*}	<0.0001	<0.0001	<0.0001
Methionine	32 ± 4^{B}	30 ± 4^{e}	32 ± 4^{B}	31 ± 4^{e}	35 ± 4^{AB}	46 ± 4^{b*}	33 ± 4^{B}	66 ± 11^{cde}	34 ± 4^{B}	41 ± 4^{bcd*}	0.02	0.003	0.003
Phenylalanine	77 ± 5	74 ± 5^{ab}	80 ± 5	78 ± 5^{ab}	72 ± 5	80 ± 5^{a}	78 ± 5	81 ± 5^{a}	78 ± 5	80 ± 5^{ab}	0.87	0.81	0.79
Threonine	176 ± 27^{CD}	166 ± 27^{c}	178 ± 26^{CD}	183 ± 26^{c}	236 ± 27^{AB}	428 ± 27^{ab*}	176 ± 24^{CD}	186 ± 24^{c}	219 ± 24^{BC}	412 ± 26^{b*}	<0.0001	<0.0001	<0.0001
Tryptophan	97 ± 7	89 ± 7^{abcd}	91 ± 7	84 ± 7^{cd}	91 ± 7	97 ± 7^{ab}	96 ± 6	95 ± 6^{abc}	92 ± 6	90 ± 7^{abcd}	0.74	0.36	0.45
Valine	301 ± 21	293 ± 21^{abc}	318 ± 20	299 ± 20^{abc}	283 ± 21	307 ± 21^{ab}	306 ± 20	314 ± 20^{a}	302 ± 20	313 ± 20^{ab}	0.44	0.54	0.81
Dispensable Amino Acids, µmol/L													
Alanine	244 ± 22^{AB}	257 ± 22^{d}	258 ± 21^{AB}	263 ± 2^{cd}	233 ± 22^{B}	313 ± 22^{bc*}	244 ± 21^{AB}	296 ± 2^{bcd*}	253 ± 21^{AB}	326 ± 21^{ab*}	0.36	<0.0001	0.002
Arginine	105 ± 10	114 ± 10^{ab}	108 ± 9	124 ± 9^{a}	99 ± 10	116 ± 10^{ab}	106 ± 9	123 ± 9^{a}	101 ± 9	127 ± 9^{a*}	0.91	<0.0001	0.65
Asparagine	95 ± 12	105 ± 12^{bcd}	95 ± 11	92 ± 11^{d}	97 ± 12	128 ± 12^{ab*}	95 ± 11	119 ± 11^{abc*}	92 ± 11	132 ± 11^{a*}	0.67	<0.0001	0.04
Aspartate	2.2 ± 0.7	5.1 ± 0.7^{a*}	3.2 ± 0.7	3.7 ± 0.7^{bc}	2.3 ± 0.7	2.5 ± 0.7^{bc}	3.1 ± 0.6	3.8 ± 0.6^{bc}	2.9 ± 0.6	3.0 ± 0.6^{bc}	0.81	0.14	0.01

Table 4.3A Continued

Citulline	107 ± 10	93 ± 10^{abc}	101 ± 10	85 ± 10^{bc*}	106 ± 10	102 ± 10^{a}	104 ± 9	101 ± 9^{a}	100 ± 9	101 ± 10^{a}	0.89	0.05	0.5
Glutamate	19 ± 2^{AB}	28 ± 2^{a*}	20 ± 2^{AB}	18 ± 2^{cd}	17 ± 2^{B}	17 ± 2^{cd}	19 ± 2^{AB}	23 ± 2^{abc*}	22 ± 2^{A}	18 ± 2^{cd*}	0.42	0.43	<0.0001
Glutamine	380 ± 32^{BC}	436 ± 32^{bcd}	425 ± 30^{ABC}	436 ± 30^{dc}	356 ± 32^{C}	474 ± 32^{abcd*}	430 ± 29^{ABC}	516 ± 29^{a*}	429 ± 29^{AB}	513 ± 30^{ab*}	0.44	<0.0001	0.17
Glycine	447 ± 30	485 ± 30^{abc}	422 ± 29	388 ± 29^{d}	443 ± 30	542 ± 32^{ab}	432 ± 29	457 ± 27^{bcd}	455 ± 29	446 ± 29^{cd}	0.25	0.07	0.04
Ornithine	58 ± 6	59 ± 6^{ab}	58 ± 5	64 ± 5^{ab}	53 ± 6	59 ± 6^{ab}	59 ± 5	64 ± 5^{ab}	58 ± 5	68 ± 5^{ab*}	0.99	<0.0001	0.74
Proline	136 ± 17	135 ± 17^{b}	139 ± 16	149 ± 16^{ab}	125 ± 17	160 ± 17^{ab*}	126 ± 15	151 ± 15^{ab*}	123 ± 15	162 ± 16^{ab*}	0.99	<0.0001	0.24
Serine	270 ± 18	277 ± 18^{cd}	289 ± 17	291 ± 17^{bcd*}	292 ± 20	338 ± 18^{ab*}	288 ± 17	326 ± 17^{abc*}	298 ± 17	349 ± 17^{a}	0.07	0.001	0.02
Tyrosine	95 ± 8	90 ± 8^{bc}	99 ± 8	92 ± 8^{bc}	87 ± 8	101 ± 8^{ab}	94 ± 8	99 ± 8^{bc}	94 ± 8	92 ± 8^{bc}	0.89	0.27	0.42

Abbreviations: PUN, plasma urea nitrogen; (-) Con, glutamate-supplemented diet ; (+) Con, lysine, histidine and threonine-supplemented diet.
Data are presented as least square means ± standard error of the mean. N = 8.
^{ABC}Different superscripts within the row indicate an effect of treatment within the pre-feeding concentrations ($P < 0.05$).
^{abc}Different superscripts within the row indicate an effect of treatment within the post-feeding concentrations $P < 0.05$).
*Significant difference between the pre and post feeding concentration within each forage treatment ($P < 0.05$).

Table 4.3B: Main effects of treatment and time on plasma concentrations of urea nitrogen and amino acids in mature horses fed diets with either alfalfa or timothy combined with amino acid supplements.

	Treatment										P-values		
	Timothy												
Metabolite	**(-) Control**		**Lysine**		**Threonine**		**Histidine**		**(+) Control**		**Treatment**	**Time**	**Treatment * Time**
Sample Time	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**			
PUN, mmol/L	4.7 ± 0.3^{E}	4.7 ± 0.3^{d}	4.9 ± 0.3^{ED}	$5.3 \pm 0.3^{bcd*}$	5.0 ± 0.3^{ED}	5.2 ± 0.3^{cd}	5.1 ± 0.3^{CDE}	5.3 ± 0.3^{bcd}	4.9 ± 0.3^{DE}	5.0 ± 0.3^{d}	0.002	0.02	0.92
Indispensable Amino Acids, µmol/L													
Histidine	75 ± 17^{BC}	99 ± 16^{c}	80 ± 18^{ABC}	76 ± 18^{c}	80 ± 19^{ABC}	80 ± 18^{c}	73 ± 18^{BC}	$215 \pm 18^{ab*}$	102 ± 16^{A}	$194 \pm 16^{b*}$	<0.0001	<0.0001	<0.0001
Isoleucine	74 ± 6^{BC}	71 ± 6^{bc}	76 ± 6^{BC}	66 ± 6^{c}	71 ± 7^{C}	76 ± 6^{abc}	75 ± 6^{BC}	75 ± 6^{abc}	74 ± 6^{BC}	73 ± 6^{bc}	0.07	0.4	0.92
Leucine	133 ± 10^{B}	125 ± 10^{bc}	139 ± 11^{B}	116 ± 11^{c}	136 ± 11^{B}	139 ± 11^{abc}	138 ± 11^{B}	133 ± 11^{abc}	137 ± 10^{B}	135 ± 10^{abc}	0.02	0.06	0.94
Lysine	85 ± 24^{B}	87 ± 22^{b}	97 ± 25^{AB}	$377 \pm 25^{a*}$	80 ± 27^{B}	99 ± 25^{b}	89 ± 25^{B}	107 ± 25^{b}	128 ± 22^{A}	$324 \pm 22^{a*}$	<0.0001	<0.0001	<0.0001
Methionine	33 ± 4^{B}	33 ± 4^{de}	37 ± 4^{AB}	33 ± 4^{de}	39 ± 4^{AB}	$55 \pm 4^{a*}$	37 ± 4^{AB}	40 ± 4^{cb}	42 ± 4^{A}	44 ± 4^{b}	0.02	0.003	0.003
Phenylalanine	74 ± 5	70 ± 5	73 ± 5	67 ± 5	77 ± 6	81 ± 5	76 ± 5	78 ± 5	77 ± 5	76 ± 5	0.87	0.81	0.79
Threonine	178 ± 26^{CD}	223 ± 24^{c}	159 ± 29^{D}	159 ± 29^{c}	270 ± 29^{A}	$494 \pm 27^{a*}$	175 ± 27^{D}	197 ± 27^{c}	252 ± 24^{AB}	$410 \pm 24^{b*}$	<0.0001	<0.0001	<0.0001
Tryptophan	85 ± 7	78 ± 6	91 ± 7	84 ± 7	93 ± 7	101 ± 7	90 ± 7	95 ± 7	88 ± 6	83 ± 6	0.74	0.36	0.45
Valine	264 ± 20	266 ± 20^{bc}	271 ± 21	252 ± 21^{c}	266 ± 23	290 ± 21^{abc}	269 ± 21	283 ± 21^{abc}	281 ± 20	285 ± 20^{abc}	0.44	0.54	0.81
Dispensable Amino Acids, µmol/L													
Alanine	262 ± 21^{AB}	286 ± 21^{bcd}	291 ± 22^{A}	287 ± 23^{bcd}	265 ± 26^{AB}	$373 \pm 24^{a*}$	263 ± 22^{AB}	$332 \pm 22^{ab*}$	279 ± 21^{AB}	$338 \pm 21^{ab*}$	0.36	<0.0001	0.002
Arginine	96 ± 9	100 ± 9^{b}	100 ± 10	114 ± 10^{ab}	93 ± 10	$128 \pm 10^{a*}$	101 ± 10	$123 \pm 10^{a*}$	99 ± 9	$122 \pm 9^{a*}$	0.91	<0.0001	0.65
Asparagine	87 ± 11	97 ± 11^{cd}	94 ± 12	97 ± 12^{c}	94 ± 12	$132 \pm 12^{a*}$	92 ± 12	$130 \pm 12^{a*}$	93 ± 11	$123 \pm 11^{ab*}$	0.67	<0.0001	0.04
Aspartate	3.4 ± 0.7	4.3 ± 0.6^{ab}	3.0 ± 0.7	2.1 ± 0.7^{c}	3.8 ± 0.8	2.6 ± 0.7^{bcd}	3.1 ± 0.7	2.9 ± 0.7^{bc}	3.4 ± 0.6^{A}	3.3 ± 0.6^{bc}	0.81	0.14	0.01

Table 4.3B Continued

Citulline	90 ± 10	83 ± 9^{bc}	92 ± 10	82 ± 10^{c}	97 ± 10	100 ± 10^{a}	98 ± 10	102 ± 10^{a}	94 ± 10	94 ± 9^{ab}	0.89	0.05	0.5
Glutamate	19 ± 2 ^{AB}	24 ± 2^{ab*}	20 ± 2^{AB}	15 ± 2^{d*}	21 ± 2^{AB}	21 ± 2^{bcd}	19 ± 2^{AB}	21 ± 2^{bcd}	21 ± 2^{AB}	18 ± 2^{d}	0.42	0.43	<0.0001
Glutamine	430 ± 30^{AB}	454 ± 29^{bcd}	416 ± 32^{ABC}	422 ± 32^{d}	414 ± 34^{ABC}	498 ± 32^{abc*}	410 ± 32^{ABC}	517 ± 32^{a*}	457 ± 29^{A}	498 ± 29^{abc}	0.44	<0.0001	0.17
Glycine	432 ± 29	428 ± 27^{cd}	463 ± 30	442 ± 30^{d}	456 ± 35	553 ± 30^{a}	446 ± 30	471 ± 30^{bcd}	456 ± 27	424 ± 27^{cd}	0.25	0.07	0.04
Ornithine	55 ± 5	58 ± 5^{b}	55 ± 6	62 ± 6^{ab}	53 ± 6	65 ± 6^{ab*}	55 ± 6	66 ± 6^{ab*}	57 ± 5	67 ± 5^{a*}	0.99	<0.0001	0.74
Proline	134 ± 16	132 ± 15^{b}	136 ± 17	135 ± 17^{b}	139 ± 17	174 ± 17^{a*}	133 ± 17	169 ± 17^{a*}	131 ± 15	154 ± 15^{ab}	0.99	<0.0001	0.24
Serine	290 ± 17	286 ± 17^{cd}	288 ± 18	245 ± 18^{d*}	291 ± 20	358 ± 18^{a*}	276 ± 18	327 ± 18^{abc*}	293 ± 17	319 ± 17^{abc}	0.07	0.001	0.02
Tyrosine	84 ± 8	87 ± 8	94 ± 8	93 ± 8	94 ± 9	114 ± 8	96 ± 8	97 ± 8	99 ± 8	99 ± 8	0.89	0.27	0.42

Abbreviations: PUN, plasma urea nitrogen; (-) Con, glutamate-supplemented diet ; (+) Con, lysine, histidine and threonine-supplemented diet.
Data are presented as least square means ± standard error of the mean. N = 8.
^{ABC}Different superscripts within the row indicate an effect of treatment within the pre-feeding concentrations ($P < 0.05$).
^{abc}Different superscripts within the row indicate an effect of treatment within the post-feeding concentrations $P < 0.05$).
*Significant difference between the pre and post feeding concentration within each forage treatment ($P < 0.05$).

Table 4.4: Main effects of forage type and amino acid supplement on 0-minute, pre-feeding plasma concentrations of urea nitrogen and amino acids in growing horses fed diets with either alfalfa or timothy combined with amino acid supplements.

Treatment													
	Alfalfa					Timothy					*P*-values		
Metabolite	**(-) Control**	**Lysine**	**Threonine**	**Histidine**	**(+) Control**	**(-) Control**	**Lysine**	**Threonine**	**Histidine**	**(+) Control**	**Forage**	**AA**	**Forage *AA**
PUN, mmol/L	6.1 ± 0.3^{A}	5.7 ± 0.3^{B}	5.9 ± 0.3^{AB}	6.0 ± 0.3^{AB}	5.9 ± 0.3^{AB}	4.6 ± 0.3*	4.8 ± 0.3*	4.8 ± 0.3*	4.9 ± 0.3*	4.8 ± 0.3*	<0.0001	0.71	0.44
Indispensable Amino Acids, µmol/L													
Histidine	71 ± 13	91 ± 13	65 ± 13	83 ± 13	84 ± 13	109 ± 13	82 ± 13	77 ± 13	76 ± 13	102 ± 13	0.15	0.32	0.20
Isoleucine	88 ± 12	115 ± 12	80 ± 12	88 ± 12	88 ± 12	93 ± 12	77 ± 12	70 ± 13	75 ± 12	75 ± 12	0.06	0.38	0.45
Leucine	158 ± 19	199 ± 19	145 ± 19	155 ± 19	166 ± 19	171 ± 19	140 ± 19	132 ± 21	137 ± 19	138 ± 19	0.06	0.38	0.35
Lysine	97 ± 33	116 ± 33	83 ± 33	140 ± 33	104 ± 33	105 ± 33	174 ± 33	80 ± 35	88 ± 33	132 ± 33	0.70	0.38	0.51
Methionine	31 ± 5	39 ± 5	34 ± 5	33 ± 5	33 ± 5	41 ± 5	37 ± 5	39 ± 5	37 ± 5	42 ± 5	0.05	0.92	0.62
Phenylalanine	78 ± 11	97 ± 11	72 ± 11	80 ± 11	78 ± 11	95 ± 11	74 ± 11	74 ± 11	76 ± 11	78 ± 11	0.78	0.56	0.31
Threonine	175 ± 25^{C}	202 ± 25^{ABC}	229 ± 25^{A}	181 ± 25^{BC}	222 ± 25^{AB}	207 ± 25^{Z}	192 ± 25^{Z}	274 ± 26^{Y}	172 ± 25^{Z}	260 ± 25^{Y}	0.15	0.002	0.54
Tryptophan	95 ± 10	97 ± 10	88 ± 10	96 ± 10	92 ± 10	107 ± 10	88 ± 10	89 ± 10	88 ± 10	89 ± 10	0.76	0.55	0.64
Valine	302 ± 43	395 ± 43	281 ± 43	309 ± 43	306 ± 43	351 ± 43	276 ± 43	263 ± 46	265 ± 43	282 ± 43	0.21	0.44	0.30
Dispensable Amino Acids, µmol/L													
Alanine	242 ± 35	309 ± 35	223 ± 35	244 ± 35	252 ± 35	343 ± 35	290 ± 35	262 ± 37	257 ± 35	286 ± 35	0.10	0.29	0.41
Arginine	107 ± 15	130 ± 15	96 ± 15	112 ± 15	103 ± 15	125 ± 15	102 ± 15	92 ± 16	99 ± 15	99 ± 15	0.45	0.42	0.52
Asparagine	97 ± 17	112 ± 17	95 ± 17	93 ± 17	92 ± 17	126 ± 17	95 ± 17	88 ± 18	91 ± 17	93 ± 17	0.96	0.52	0.56
Aspartate	2.5 ± 0.7	4.0 ± 0.7	2.6 ± 0.7	2.9 ± 0.7	2.9 ± 0.7	4.1 ± 0.7	3.4 ± 0.7	4.2 ± 0.8	3.3 ± 0.7	3.0 ± 0.7	0.11	0.76	0.31
Citrulline	19 ± 3	27 ± 3	17 ± 3	18 ± 3	22 ± 3	25 ± 3	21 ± 3	24 ± 4	20 ± 3	20 ± 3	0.59	0.63	0.28
Glutamate	401 ± 42	466 ± 42	363 ± 42	432 ± 42	434 ± 42	518 ± 42	434 ± 42	423 ± 45	414 ± 42	468 ± 42	0.19	0.42	0.30

Table 4.4 Continued

Glutamine	436 ± 48	508 ± 48	433 ± 48	446 ± 48	485 ± 48	525 ± 48	445 ± 48	479 ± 51	430 ± 48	460 ± 48	0.81	0.86	0.44
Glycine	133 ± 27	180 ± 27	117 ± 27	125 ± 27	124 ± 27	185 ± 27	134 ± 27	131 ± 29	126 ± 27	136 ± 27	0.65	0.35	0.35
Ornithine	274 ± 29	329 ± 29	270 ± 29	279 ± 29	300 ± 29	355 ± 29	288 ± 29	292 ± 31	277 ± 29	301 ± 29	0.50	0.61	0.29
Proline	97 ± 13	120 ± 13	88 ± 13	93 ± 13	95 ± 13	109 ± 13	95 ± 13	91 ± 14	96 ± 13	99 ± 13	0.98	0.56	0.58
Serine	71 ± 13	91 ± 13	65 ± 13	83 ± 13	84 ± 13	109 ± 13	82 ± 13	77 ± 13	76 ± 13	102 ± 13	0.15	0.32	0.20
Tyrosine	88 ± 12	115 ± 12	80 ± 12	88 ± 12	88 ± 12	93 ± 12	77 ± 12	70 ± 13	75 ± 12	75 ± 12	0.06	0.38	0.45

Abbreviations: PUN, plasma urea nitrogen; (-) Con, glutamate-supplemented diet ; (+) Con, lysine, histidine and threonine-supplemented diet.
Data are presented as least square means ± standard error of the mean. N = 8.
[ABC]Different superscripts within the row indicate an effect of treatment within the alfalfa-based diets ($P < 0.05$).
[YZ]Different superscripts within the row indicate an effect of treatment within the timothy-based diets $P < 0.05$).
[*]Significant difference between forage type within each AA supplement ($P < 0.05$).

Table 4.5: Main effects of forage type and amino acid supplement on 90-minute, post-feeding plasma concentrations of urea nitrogen and amino acids in growing horses fed a diet with either alfalfa or timothy combined with amino acid supplements.

	Treatment												
	Alfalfa					Timothy					*P*-values		
Metabolite	**(-) Control**	**Lysine**	**Threonine**	**Histidine**	**(+) Control**	**(-) Control**	**Lysine**	**Threonine**	**Histidine**	**(+) Control**	**Forage**	**AA**	**Forage * AA**
PUN, mmol/L	6.0 ± 0.3	6.0 ± 0.3	6.1 ± 0.3	6.1 ± 0.3	6.1 ± 0.3	4.6 ± 0.3^{X}	5.1 ± 0.3^{W}	4.9 ± 0.3^{WX}	5.1 ± 0.3^{W}	4.8 ± 0.3^{WX}	<.0001	0.51	0.38
Indispensable Amino Acids, µmol/L													
Histidine	77 ± 18^{B}	83 ± 18^{B}	83 ± 18^{B}	212 ± 18^{A}	233 ± 18^{A}	82 ± 18^{X}	78 ± 18^{X}	81 ± 18^{X}	230 ± 18^{W}	227 ± 18^{W}	0.83	<.0001	0.89
Isoleucine	82 ± 5	86 ± 5	87 ± 5	89 ± 5	84 ± 5	71 ± 5	67 ± 5*	74 ± 5*	76 ± 5*	70 ± 5*	<.0001	0.59	0.92
Leucine	146 ± 9	155 ± 9	159 ± 9	158 ± 9	150 ± 9	125 ± 9	118 ± 9*	133 ± 9*	134 ± 9*	132 ± 9	<.0001	0.57	0.81
Lysine	91 ± 26^{B}	364 ± 26^{A}	100 ± 26^{B}	106 ± 26^{B}	350 ± 28^{A}	88 ± 26^{X}	341 ± 26^{W}	96 ± 26^{X}	107 ± 26^{X}	388 ± 26^{W}	0.90	<.0001	0.85
Methionine	30 ± 15	29 ± 15	47 ± 15	70 ± 15	73 ± 16	33 ± 15	32 ± 15	53 ± 15	36 ± 15	44 ± 15	0.29	0.22	0.48
Phenylalanine	75 ± 4	79 ± 4	82 ± 4	83 ± 4	77 ± 4	71 ± 4	67 ± 4*	78 ± 4	79 ± 4	76 ± 4	0.04	0.09	0.70
Threonine	167 ± 23^{B}	160 ± 23^{B}	429 ± 23^{A}	193 ± 23^{B}	436 ± 25^{A}	198 ± 23^{Y}	147 ± 23^{Z}	486 ± 23^{W}	194 ± 23^{YZ}	430 ± 23	0.33	<.0001	0.50
Tryptophan	88 ± 6	86 ± 6	95 ± 6	95 ± 6	87 ± 6	78 ± 6^{Y}	81 ± 6^{Y}	95 ± 6^{W}	93 ± 6^{WX}	82 ± 6^{XY}	0.15	0.008	0.83
Valine	294 ± 17	303 ± 17	311 ± 17	318 ± 17	308 ± 18	266 ± 17	255 ± 17*	285 ± 17	282 ± 17	280 ± 17	0.0004	0.40	0.94
Dispensable Amino Acids, µmol/L													
Alanine	255 ± 22^{B}	257 ± 22^{B}	310 ± 22^{A}	296 ± 22^{A}	315 ± 23^{A}	288 ± 22^{XY}	258 ± 22^{Y}	341 ± 22^{W}	326 ± 22^{WX}	341 ± 22^{W}	0.03	0.0001	0.88
Arginine	109 ± 8	128 ± 8	119 ± 8	125 ± 8	125 ± 9	103 ± 8	109 ± 8	114 ± 8	121 ± 8	121 ± 8	0.10	0.12	0.78
Asparagine	101 ± 11^{B}	96 ± 11^{B}	133 ± 11^{A}	125 ± 11^{A}	129 ± 11^{A}	97 ± 11^{X}	93 ± 11^{X}	125 ± 11^{W}	129 ± 11^{W}	120 ± 11^{W}	0.42	<.0001	0.95
Aspartate	5.3 ± 0.7^{A}	3 ± 0.7^{B}	3.1 ± 0.7^{B}	4.8 ± 0.7^{A}	2.9 ± 0.7^{B}	3.9 ± 0.7^{W}	2.1 ± 0.7^{X}	3.3 ± 0.7^{W}	3.2 ± 0.7^{WX*}	3 ± 0.7^{WX}	0.05	0.002	0.30
Citrulline	91 ± 8^{BC}	82 ± 8^{C}	100 ± 8^{AB}	104 ± 8^{A}	99 ± 8^{AB}	83 ± 8^{X}	80 ± 8^{X}	96 ± 8^{W}	99 ± 8^{W}	94 ± 8^{W}	0.11	<.0001	0.98
Glutamate	47 ± 7^{A}	16 ± 7^{B}	18 ± 7^{B}	25 ± 7^{B}	18 ± 7^{B}	23 ± 7^{W}	16 ± 7^{X}	22 ± 7^{W}	21 ± 7^{W}	17 ± 7^{X}	0.27	0.04	0.27
Glutamine	449 ± 29^{BC}	445 ± 29^{C}	488 ± 29^{AB}	542 ± 29^{A}	501 ± ^{30AB}	448 ± 29^{XY}	417 ± 29^{Y}	490 ± 29^{WX}	532 ± 29^{W}	500 ± 29^{W}	0.56	<.0001	0.94
Glycine	470 ± 36^{B}	404 ± 36^{B}	565 ± 36^{A}	461 ± 36^{B}	455 ± 38^{B}	428 ± 36^{X}	430 ± 36^{X}	519 ± 36^{W}	453 ± 36^{X}	423 ± 36^{X}	0.26	0.0004	0.68
Ornithine	59 ± 4	65 ± 4	60 ± 4	66 ± 4	66 ± 5	58 ± 4	60 ± 4	62 ± 4	67 ± 4	68 ± 4	0.87	0.06	0.82
Proline	132 ± 16^{B}	145 ± 16^{AB}	161 ± 16^{A}	157 ± 16^{A}	155 ± 17^{AB}	136 ± 16^{WX}	126 ± 16^{X}	163 ± 16^{W}	162 ± 16^{W}	149 ± 16^{WX}	0.70	0.02	0.78

Table 4.5 Continued

Serine	287 ± 17^{B}	276 ± 17^{B}	339 ± 17^{A}	337 ± 17^{A}	340 ± 18^{A}	282 ± 17^{X}	243 ± 17^{Y}	348 ± 17^{W}	333 ± 17^{W}	322 ± 17^{W}	0.33	<.0001	0.76
Tyrosine	92 ± 6^{B}	95 ± 6^{AB}	104 ± 6^{A}	98 ± 6^{AB}	89 ± 6^{B}	89 ± 6^{X}	91 ± 6^{X}	109 ± 6^{W}	99 ± 6^{WX}	96 ± 6^{X}	0.71	0.03	0.79

Abbreviations: PUN, plasma urea nitrogen; (-) Con, glutamate-supplemented diet ; (+) Con, lysine, histidine and threonine-supplemented diet.
Data are presented as least square means ± standard error of the mean. N = 8.
[ABC]Different superscripts within the row indicate an effect of treatment within the alfalfa-based diets ($P < 0.05$).
[YZ]Different superscripts within the row indicate an effect of treatment within the timothy-based diets $P < 0.05$).
[*]Significant difference between forage type within each supplement ($P < 0.05$).

Table 4.6: Effect of treatment on parameters of phenylalanine flux in mature horses fed diets with either alfalfa or timothy supplemented with glutamate ((-) Con), lysine, threonine, histidine, or lysine, threonine and histidine ((+) Con), µmol/(kg·h)

	Alfalfa					Timothy					Pooled SEM	P-value
Measured Parameter	**(-) Con**	**Lys**	**Thr**	**His**	**(+) Con**	**(-) Con**	**Lys**	**Thr**	**His**	**(+) Con**		**Treatment**
Phenylalanine flux[1]	66^{AB}	74^{A}	66^{AB}	72^{A}	66^{AB}	62^{B}	61^{B}	61^{B}	59^{B}	61^{B}	2	<0.0001
Phenylalanine oxidation[2]	23^{ABC}	24^{ABC}	25^{A}	24^{AB}	23^{ABC}	18^{C}	19^{BC}	18^{BC}	20^{ABC}	20^{ABC}	1	0.0004
Phenylalanine for protein synthesis[3]	43^{AB}	50^{A}	41^{AB}	48^{AB}	43^{AB}	44^{AB}	43^{AB}	43^{AB}	39^{B}	41^{AB}	3	0.05
Dietary Phenylalanine Intake[4]	9.0^{A}	9.0^{A}	9.0^{A}	9.0^{A}	9.0^{A}	8.0^{B}	8.0^{B}	8.0^{B}	8.0^{B}	8.0^{B}	0.1	<0.0001
Phenylalanine from protein breakdown[5]	33	41	32	39	34	36	35	35	32	34	3	0.13
CO_2 production[6]	14364	15608	15366	15760	15493	14025	14292	13699	15168	14966	669	0.13

[1] Flux = isotope infusion rate × [(isotope solution enrichment/ plateau plasma enrichment)-1].
[2] Oxidation = 100 × [(1/plateau plasma enrichment – 1/isotope solution enrichment) × rate of $^{13}CO_2$ release during phenylalanine infusion].
[3] Phenylalanine for protein synthesis = Non-oxidative Phenylalanine disposal = Flux – Oxidation.
[4] Assumes a pre-cecal digestibility of 42% (Gibbs et al., 1988; Farley et al., 1995) and a splanchnic extraction of 26.5% (Mastellar et al., 2016a).
[5] Phenylalanine entering pool from protein degradation = Flux – Phenylalanine from diet.
[6] Total carbon dioxide production = isotope infusion rate × [(isotope solution enrichment/ plateau breath enrichment)-1] × [0.0224 mL/µmol CO_2]
^{ABC} indicate differences between treatments.
(-) Con, glutamate-supplemented diet; Lys, lysine-supplemented diet; Thr, threonine-supplemented diet; His, histidine-supplemented diet; (+) Con, lysine, histidine and threonine-supplemented diet.

Table 4.7: Effect of forage type or of amino acid supplementation on parameters of phenylalanine flux in mature horses fed diets with either alfalfa or timothy supplemented with glutamate ((-) Con), lysine, threonine, histidine, or lysine, threonine and histidine ((+) Con), μmol/(kg·h)

	Alfalfa					Timothy					Pooled SEM	P-values		
Measured Parameter	**(-) Con**	**Lys**	**Thr**	**His**	**(+) Con**	**(-) Con**	**Lys**	**Thr**	**His**	**(+) Con**		**Forage**	**Amino Acid**	**Forage * AA**
Phenylalanine flux[1]	66	74	66	72	66	62	61*	61	59*	61	2	0.0025	0.15	0.04
Phenylalanine oxidation[2]	23	24	25	24	23	18	19	18*	20	20	1	0.0008	0.70	0.59
Phenylalanine for protein synthesis[3]	43	50	41	48	43	44	43	43	39	41	3	0.21	0.25	0.05
Dietary Phenylalanine Intake[4]	9.0	9.0	9.0	9.0	9.0	8.0*	8.0*	8.0*	8.0*	8.0*	0.1	<0.0001	0.89	0.96
Phenylalanine from protein breakdown[5]	33	41	32	39	34	36	35	35	32	34	3	0.59	0.25	0.05
CO_2 production[6]	14364	15608	15366	15760	15493	14025	14292	13699	15168	14966	669	0.19	0.15	0.71

[1] Flux = isotope infusion rate × [(isotope solution enrichment/ plateau plasma enrichment)-1].
[2] Oxidation = 100 × [(1/plateau plasma enrichment – 1/isotope solution enrichment) × rate of $^{13}CO_2$ release during phenylalanine infusion].
[3] Phenylalanine for protein synthesis = Non-oxidative Phenylalanine disposal = Flux – Oxidation.
[4] Assumes a pre-cecal digestibility of 42% (Gibbs et al., 1988; Farley et al., 1995) and a splanchnic extraction of 26.5% (Mastellar et al., 2016a).
[5] Phenylalanine entering pool from protein degradation = Flux – Phenylalanine from diet.
[6] Total carbon dioxide production = isotope infusion rate × [(isotope solution enrichment/ plateau breath enrichment)-1] × [0.0224 mL/μmol CO_2].
* indicates significant main effect of forage on the timothy sample that is different from the alfalfa sample within an amino acid supplement.
(-) Con, glutamate-supplemented diet; Lys, lysine-supplemented diet; Thr, threonine-supplemented diet; His, histidine-supplemented diet; (+) Con, lysine, histidine and threonine-supplemented diet.

Chapter 5: Effect of forage type and ration balancer protein content on measures of whole-body protein metabolism in growing horses consuming a predominantly forage diet

Abstract

Predominantly forage diets are able to fulfill energy and protein requirements of horses at maintenance or light work. However, little research has been done investigating the effects of forage-based diets in growing horses. Forage type and maturity influences protein content, and typically legume hays contain a higher crude protein percentage than grass hays. Ration balancers are used to provide additional nutrients to a diet, such as protein, minerals or vitamins, without drastically increasing the caloric load. The objective of this study was to determine the effects of either timothy or alfalfa hay consumption supplemented with either a high or low protein ration balancer on measures of whole-body protein metabolism. Eight Thoroughbred yearlings (aged 9.55 ± 0.53 months; 332.8 ± 18.95 kg, BW), 4 colts and 4 fillies, were fed 2% of body weight per day of either a timothy (10.4%, CP) or alfalfa hay (17.5%, CP) supplemented with 0.7% of their BW per day of either a high (30.5%, CP) or low protein (8.5%, CP) ration balancer for 14 days. All horses were studied while receiving all treatments. After adapting to each treatment for 13 days, blood samples were collected by jugular venipuncture pre and 90 minutes post feeding the morning meal. The blood plasma was analyzed for amino acid concentrations using HPLC and urea nitrogen concentrations using a spectrophotometric assay. On day 14, isotope infusion procedures included a 2h primed, constant intravenous infusion of [^{13}C]sodium bicarbonate to measure CO_2 production followed by a 4h primed, constant intravenous infusion of [1-^{13}C]phenylalanine, for measurement of phenylalanine oxidation and estimated rates of non-oxidative phenylalanine disposal. Blood and breath samples were collected throughout the isotope infusion to measure plasma [^{13}C]phenylalanine enrichment, using GC-MS, and breath $^{13}CO_2$ enrichment, using an infrared isotope analyzer. Data were analyzed using PROC GLIMMIX procedure of SAS version 9.2, with treatment, time and the interaction between the two variables as the fixed effects. There was no effect of treatment on average daily gain ($P = 0.18$). Plasma urea nitrogen concentrations were lower when horses consumed the timothy-based diets ($P < 0.0001$) and when horses consumed the low protein ration balancer diets ($P < 0.0001$). Horses had greater plasma concentrations of all the branched chain amino acids (BCAA), isoleucine ($P = 0.01$) leucine ($P = 0.0008$) and valine ($P < 0.0001$), after consuming the alfalfa-based diets compared to timothy-based diets. After horses consumed the high protein ration balancer, plasma BCAA concentrations were greater than when horses consumed the low protein ration balancer ($P < 0.05$). Phenylalanine flux ($P = 0.001$) oxidation ($P = 0.004$) and use for protein synthesis ($P = 0.009$) were greater when horses consumed the alfalfa-based diets vs the timothy-based diets. These results suggest that yearling horses are able to achieve greater rates of whole-body protein synthesis when fed alfalfa-based diets, as compared to those receiving timothy.

Introduction

As herbivores, the horse's digestive tract is designed to receive most of its required nutrients from forage. However, the nutritional value of forage is highly dependent on the type and quality of the forage as well as the physiological status and age of the horse. There are two key types of forage fed to horses, grass and legumes. An example of a common legume fed to horses is alfalfa, which tends to have greater digestible energy and crude protein than grasses. Forage management and growing conditions also influence the nutrient availability of forages. To support adequate growth, weanlings and yearlings have higher nutrient requirements than mature horses. Requirements for dietary protein are provided in terms of crude protein, which is an estimate based on the nitrogen content in feed (NRC, 2007). Typically, energy and crude protein are the two most limiting factors in the diet of growing horses (NRC, 2007). Moreover, on a body weight basis, crude protein requirements for growing horses are second only to the lactating mare (NRC, 2007). To achieve optimal growth rates in yearling horses, it has been previously suggested that the diet needs to supply a minimal level of 13-17% of crude protein (CP), to meet the daily requirements of 1015g of protein per day for a yearling horse estimated to weigh 600kg at maturity (Jordon and Myers, 1972; Schryver et al., 1987; NRC 2007). A good quality forage can easily meet or exceed the protein requirements to support growth, but other nutrients, such as energy, may be deficient (Rouquette et al., 1985; Hansen et al., 1987; NRC, 2007). To ensure that requirements for energy and other nutrients are met, horses are commonly fed protein in excess of daily needs (Gallagher et al., 1992a; Gallagher et al., 1992b; Honoré and Uhlinger, 1994; Harper et al., 2009).

When amino acid requirements exceed the animal's requirements for the most limiting amino acid, they are catabolized and destined for excretion. During catabolism, the carbon backbones of the amino acids are converted to CO_2 and marked for exhalation, and the amine groups are cleaved and converted to urea via the hepatic urea cycle, which is then excreted in urine. When amino acid catabolism increases, urea-nitrogen excretion from the urine increases, which can lead to greater levels of ammonia in the atmosphere (Weir et al., 2017). The largest source of ammonia emissions in the Unites States is animal agriculture (USEPA, 2005). Ammonia is an air and water pollutant that contributes to eutrophication, soil acidity and aerosol formation, which can negatively impact the environment and health (Hristov et al., 2011).

Studies have been conducted in growing horses to investigate the effects of amino acid supplementation in the diet in optimizing protein synthesis and reducing waste (Hintz et al., 1971b; Graham et al., 1994; Staniar et al., 2001; Yoshiyuki et al., 2007; Tanner et al., 2014; Mastellar et al., 2016a; Mastellar et al., 2016b). Early work in growing horses found that lysine supplementation to a linseed meal diet clearly improved growth as well as the feed to gain ratio (Hintz et al., 1971b). Threonine has also been evaluated in yearling horses as a potentially limiting amino acid (Graham et al., 1994). A reduction in serum urea nitrogen concentrations and improved growth was seen in yearlings receiving concentrate supplemented with lysine and threonine compared to yearlings receiving only concentrate. This study established that by improving protein quality, the overall concentration of CP can be reduced in the diet (Graham et al., 1994).

The objective of the present study was to investigate whether whole-body protein metabolism of growing horses is affected by either alfalfa or timothy grass hay

consumption or the protein content of the ration balancer, when horses received primarily forage-based diets. It was hypothesized that if a forage source was limiting in one or more indispensable amino acids, then protein quality would be improved by the addition of the high protein ration balancer, resulting in greater rates of whole-body protein synthesis.

Materials and methods

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals, Housing, and Feeding

Four female and four intact male Thoroughbred horses initially aged 9.55 ± 0.53 months were obtained from the University of Kentucky Department of Animal and Food Sciences' research herd. Initially, horse's body weights ranged from 299.5 kg to 354.5 kg, with an average weight of 332.8 ± 19.0 kg (mean ± standard deviation). Horses were weighed using an electronic scale (TI-500, Transcell Technology Inc., Buffalo Grove, IL). They were all housed individually in 3.7 × 3.7 m stalls overnight. Between the hours of 0800 and 1500, horses were turned out in two, 0.25-acre paddocks, separated by gender, and fitted with muzzles to prevent grazing. Horses were fed their experimental diets at 0700 and 1500, any feed refusals were collected after the morning meal and weighed each day to calculate total daily intake.

Experimental Design

Horses were divided into two groups, with two fillies and two colts in each group, and were randomly assigned to 1 of 2 forage treatments (alfalfa or timothy hay), described below, in a randomized crossover design. For each forage group horses were

allocated to receive one of two ration balancers (with either a low or high protein level), also in a crossover design. For 7 days prior to the first period and treatment, horses were individually housed in stalls and slowly adapted to all of the hay and concentrate combinations, as well as to handling and daily turnout procedures. On day 0 of each period, horses were fed their first experimental diet as the afternoon meal and continued to consume that diet for 14 days prior to collection. On day 13, pre- and post- feeding blood samples were taken to determine plasma urea nitrogen and amino acid concentrations, and on day 14, the isotope infusion protocol was used. These procedures will be outlined in greater detail below in the sampling and isotope infusion section. Horses were then switched to the second dietary treatment period on the afternoon of day 14 after infusion procedures were concluded. A washout period of 14 days was used between periods 2 and 3 to allow for horses to adjust from either timothy hay to alfalfa hay or vice versa.

Dietary Treatments

Horses received each of four dietary treatments in a randomized crossover design. Treatments were a combination of either alfalfa or timothy hay, and a ration balancer that was either high or low in crude protein. The first treatment was alfalfa hay and the high protein ration balancer. The second treatment was alfalfa hay and the low protein ration balancer. The third treatment was timothy hay and the high protein ration balancer. The fourth and final treatment was timothy hay and the low protein ration balancer. The analyzed nutrient compositions of the dietary components are shown in Table 5.1. All experimental diets were formulated to meet, or exceed, the estimated requirements for digestible energy (DE), CP and vitamin and minerals (NRC, 2007).

Hay was always offered at 2.0% of bodyweight on an as-fed basis and the ration balancer cube was fed at 0.7% of BW per day on sample collection days (days 13-14). On non-sample collection days (days 0-12), the ration balancer cube was still always fed at 0.7% of BW per day, but when horses consumed over 85% of the hay offered to them the night before, their hay allocation was raised by 0.1% of bodyweight on an as-fed basis to allow for maximal forage intake. By the end of the study, all horses were offered at least 2.2% of their bodyweight on an as-fed basis as hay, with one horse offered up to 2.5% of their bodyweight on an as-fed basis of hay. The ration balancer high in crude protein (30.5%, CP) was a commercially available concentrate (M30, McCauleys Bros, Versailles, KY). The ration balancer low in crude protein (8.5%, CP) was formulated and manufactured specifically for this study (McCauleys Bros, Versailles, KY; Table 5.2). The low protein concentrate was formulated to be isocaloric with the high protein balancer.

Sampling and isotope infusion procedures

All animals were weighed on day 13 of each experimental period, prior to pre-prandial blood collection. Blood samples were collected on day 13 of each study period prior to the morning meal of concentrate and 1 kg of hay 90 postprandial to measure the effects of forage type and ration balancer protein content on plasma amino acid and plasma urea nitrogen concentrations. Blood was collected via venipuncture from the jugular vein into heparinized vacutainers (BD, Franklin Lakes, NJ) and immediately centrifuged at 1,500 × g for 10 min at 4 ºC. The supernatant plasma was collected and stored at -20 ºC until the time of analysis.

On the morning of day 14 of each study period, two jugular intravenous catheters were aseptically fitted, one for blood collection and the other for intravenous isotope infusion. Whole-body phenylalanine kinetics were determined using a 2 h primed, constant intravenous infusion of [^{13}C] sodium bicarbonate at a prime rate of 5.3 μmol/(kg BW·h) and a constant rate of 4.4 μmol/(kg BW·h), followed by a 4 h primed, constant intravenous administration of [1-^{13}C] phenylalanine at a prime rate of 12.6 μmol/(kg BW·h) and a constant rate of 9.0 μmol/(kg BW·h) (Cambridge Isotope Laboratories, Andover, MA), as previously described (Urschel et al., 2012; Masteller et al., 2016a). The prime (1.4:1 for phenylalanine and 1.2:1 for bicarbonate) to constant ratios for both isotopes were previously validated in horses (Urschel et al., 2012), and the dose of phenylalanine has been shown to result in stable, measurable plateaus of isotope within the breath (Mastellar et al., 2016a). The goal of bicarbonate isotope infusion was to estimate total CO_2 production (Hoerr et al., 1989), whereas the phenylalanine isotope infusion allowed for the measurement of phenylalanine oxidation to CO_2 and phenylalanine flux, which enabled the subsequent estimates of rates of whole-body protein synthesis and degradation.

To maintain a steady metabolic state, horses received 1/48th of their daily ration balancer meal every half hour throughout sampling, beginning 90 minutes before the start of the bicarbonate infusion. In addition to the ration balancer meal, 1/48th of 2.0% of bodyweight was provided as whichever respective forage type the horses were allocated to during that period. Any feed refusals that remained after 30 minutes were removed and weighed. Immediately prior to isotope infusion, two baseline blood and breath samples were collected. Blood (10 mL) was collected from a catheter placed in the jugular vein

and processed as described for day 13 sampling. Breath was collected into gas impermeable bags using a modified equine Aeromask (BreathEazy Ltd, Malvern, Worcestershire) and immediately analyzed, as described below (Urschel et al., 2012). Following baseline sample collection, [^{13}C] sodium bicarbonate was infused into the intravenous catheter using a cordless infusion pump (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc.) attached to a surcingle worn by the horse. During the bicarbonate isotope infusion, breath samples were collected every 30 min for the first hour, then every 15 min during the second hour. After 2 h, infusion was stopped and horses received their [1-^{13}C] phenylalanine prime dose intravenously in their catheter, and the [1-^{13}C] phenylalanine was infused into the intravenous catheter using a cordless infusion pump again. Throughout phenylalanine administration, blood and breath samples were collected every half hour. At the end of the infusion period, both catheters were removed and horses received their day 0 meal for the next treatment period. All procedures were repeated at the end of each dietary treatment period, so that each horse was studied while receiving each of the four treatments.

Sample Analysis

Pre- and post-feeding blood samples from day 13 were analyzed for plasma amino acid and urea nitrogen concentrations. A colorimetric spectrophotometric assay was used to measure plasma urea nitrogen concentrations. A 10-µL aliquot of each plasma sample was pipetted, in duplicate, into microcentrifuge tubes containing 125 µL of chilled urease buffer (Sigma-Aldrich Co., St. Louis, MO). The plasma samples were pipetted on the side of the tube, above the urease buffer, to initiate the enzymatic reaction for all samples at the same time, using a multi-tube vortexer (Scientific Manufacturing Industries,

Emeryville, CA). After 20 min of incubation in a rack on ice, 250 µL of phenol nitroprusside solution was added to all tubes with a repeat pipetter, then vortexed. Next, 250 µL of alkaline hypochlorite solution, and 1000 µL of distilled water were added to each sample, then vortexed. After 25 min of incubation at room temperature, 200 µL of each sample were transferred in duplicate to 96 well plates, placed in a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA,) and read at 570 nm of wavelength. The inter-assay variation between plates for the control sample was 6.09% and the average intra-assay variation within each plate for the control sample was 4.88%.

Using high performance liquid chromatography (HPLC) with a previously described method (Bidlingmeyer et al., 1984; Urschel et al., 2011), plasma amino acid concentrations were determined. Norleucine was added to all samples as an internal standard, then all samples were deproteinated using 10-kd cutoff centrifugal filters spun at 15,000 x g for 30 minutes at 4 ºC, and the filtrate was freeze-dried. A re-dry solution of methanol, 1mM of sodium acetate, and trimethylamine (TEA) (Fisher Scientific, Fair Lawn, NJ) were combined in a 2:2:1 ratio, and vortexed. A 10uL aliquot of re-dry solution was added to all tubes, then 25uL of a 0.2mM glutamine solution was added to standards, then all tubes were freeze dried. Amino acids were then derivatized by adding a mixture of ethanol, TEA, water, and phenylisothiocyanate (Acro Organics, Geel, Belgium) in a 7:1:1:1 ratio and incubating the samples in a sealed container at room temperature for 20 min. Samples were then freeze-dried and reconstituted with 100 uL of HPLC eluent and injected onto a 3.9x300mm Nova-Pak ® C18 4 µm reverse phase column (Waters Corporation, Milford, MA).

Samples of timothy hay, alfalfa hay, high protein ration balancer and low protein ration balancer were collected before adaptation and sent for proximate analysis to a commercial lab via wet chemistry (Dairy One Cooperative Inc., Ithaca, NY). Feed amino acid concentrations were determined using acid hydrolysis (AOAC International, 2005). Samples were ground, and 0.2 g was weighed into ashed vials. To these vials 12 mL of 6N hydrochloric acid (Fisher Scientific, Fair Lawn, NJ) was added and samples were capped tightly and incubated at 110 °C for 24 h. After incubation, samples were filtered using a 0.45 μm syringe filter (Sarstedt, Numbrecht, Germany) into microcentrifuge tubes. Samples were then derivatized and analyzed by HPLC, as described for plasma samples. To measure methionine concentrations, a separate assay was performed in which 2 mL of performic acid (J.T. Baker Chemicals, Center Valley, PA) and 0.42 g of metabisulfite (Fisher Scientific, Fair Lawn, NJ) were added to samples and incubated overnight at 4 °C prior to the addition of hydrochloric acid (AOAC International, 2005).

The isotope enrichment of plasma samples collected during isotope infusion was determined by negative chemical ionization GC-MS analysis of a heptafluorobutyric, n33 propyl derivative at a commercial laboratory (Metabolic Solutions Inc., Nashua, NH), as previously described (Matthews et al., 1990; Wagner et al., 2013). A Phenomenex ZB-1MS capillary column was used to separate the derivative of phenylalanine. Selected ion chromatograms were obtained by monitoring ions at a m/z 383 and 384 for phenylalanine and [1- ^{13}C]phenylalanine, respectively. The isotope enrichment of breath samples was determined by measuring the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the breath using an infrared isotope analyzer (IRIS-3; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany) (Urschel et al., 2012).

Calculations

For both plasma and breath samples, the average enrichment at isotopic stable state, or plateau, was determined. The plateau was defined as at least 3 points with a coefficient of variation less than 10%. If the plateau was not obtained, the data was discarded. Total CO_2 production rate was determined using the average enrichment of breath samples during [^{13}C] sodium bicarbonate infusion with the following formula:

$$CO_2 \text{ production} = i \times [Ei/Eb) -1] \times [0.0224 \text{ ml}/\mu\text{mol } CO_2]$$

where *i* is rate of the isotope infusion (μmol/(kg BW·h)), *Ei* is the enrichment of isotope solution, and *Eb* is the plateau breath enrichment (Hoerr et al., 1989).

The difference of phenylalanine entering into the free amino acid pool vs the rate of exit of phenylalanine from the free amino acid pool is coined whole-body phenylalanine flux. This flux is calculated using the following equation where Q is flux (μmol/(kg BW·h)), *i* is rate of the isotope infusion (μmol/(kg BW·h)), *Ei* is isotope solution enrichment, and *Ep* is the plateau plasma enrichment (Hsu et al., 2006):

$$Q = i \times [(Ei/Ep) -1]$$

The different processes affecting flux include amino acids entering the blood amino acid pool from dietary intake (I), *de novo* synthesis (N), and protein breakdown (B), or leaving the pool through protein synthesis (Z), oxidation (E), or the conversion to other metabolites (M) (Picou and Taylor-Roberts, 1969):

$$Q = I + N + B = Z + E + M$$

Due to a lack of research regarding phenylalanine digestibility and the splanchnic extraction of digested phenylalanine in yearling horses, the amount of phenylalanine entering the plasma amino acid pool from protein breakdown (I) could not be confidently

estimated or calculated. Therefore, the phenylalanine release from protein degradation was not able to be estimated.

Phenylalanine oxidation was calculated using the following equation (Hsu et al., 2006):

$$E = F^{13}CO_2\ (1/Ep - 1/Ei) \times 100$$

where E represents phenylalanine oxidation (μmol/ (kg BW·h)) and $F^{13}CO_2$ is the product of isotope enrichment of the breath and the rate of carbon dioxide production (μmol/ (kg BW·h)). Tyrosine was believed to be provided in excess for all treatments, therefore the conversion of phenylalanine to tyrosine (M) was considered negligible. Therefore, protein synthesis was estimated using the following equation:

$$Z = Q - E$$

Statistical Analysis

All experimental data were analyzed using the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) with significance set at $P \leq 0.05$. Measurements are presented as least squares means ± standard error of the mean, or as least squares means with a pooled standard error of the mean where applicable.

Plasma urea nitrogen and plasma amino acid concentrations were analyzed using repeated measures, with time, treatment and their interaction as the fixed effects and horse as the random subject. A heterogeneous autoregression variance-covariance structure was chosen based on lowest values for respective fit statistics (AIC).

To further analyze treatment differences within both the pre-feeding and 90 min post-feeding time points, treatments were separated into two variables: forage type

(alfalfa or timothy) and protein level of the ration balancer (either high or low protein). Plasma urea nitrogen and plasma amino acid concentrations at each timepoint were analyzed as a two-way ANOVA with forage, protein level and their interaction as the fixed effects and horse was included as a random effect.

A one-way ANOVA was used to analyze treatment effects on average daily gain and whole-body phenylalanine kinetics with treatment as the fixed effect, and horse as the random effect. To better characterize the effect of forage type and amino acid supplement on whole-body phenylalanine kinetics, this data was also analyzed as a 2x2 factorial design, with forage, ration balancer protein level and their interaction as the fixed effects and horse was included as a random effect.

For all cases, when fixed effects were significant, the means were separated using the Tukey-adjusted pdiff option.

Results

All horses remained healthy for the entire study. All horses consumed most of the experimental diet during all adaptation periods and sampling procedures. The daily nutrient intakes in each treatment have been calculated and are documented in Table 5.3. Due to behavioral issues with some of the yearlings, not all horses completed all experimental sampling procedures. When a treatment included less than the desired n= 8 horses, this will be noted on the table legend. One horse on the timothy and the low protein ration balancer treatment did not cooperate for isotopic sampling procedures and was removed from sampling that day. Another horse on the timothy and low protein

ration balancer treatment left orts of hay during the isotope infusion procedures for some sampling time points, and so prior to feeding the next allocation of feed, the orts were removed and weighed and the actual intake of hay was recorded. Additionally, one horse on the alfalfa and low protein ration balancer treatment was uncooperative for the collection of one of the post-feeding blood samples and so that sample could not be obtained. All other sampling procedures were conducted as planned in all horses.

Average daily gain

Overall, there was no significant effect of treatment on average daily gain (ADG) ($P = 0.18$). There was no effect of ration balancer protein level on ADG ($P = 0.63$), but there was a trend for greater ADGs in horses fed the alfalfa-based diets when compared to horses fed the timothy-based diets regardless of ration balancer addition ($P = 0.056$). Horses receiving the alfalfa and high protein ration balancer diet had an ADG of 0.55 ± 0.49 kg/day. The ADG for horses receiving the alfalfa and the low protein ration balancer diet was 0.62 ± 0.26 kg/day. For horses receiving the timothy and the high protein ration balancer diet, 0.45 ± 0.25 kg/day was their ADG. Lastly, horses receiving the timothy and the low protein ration balancer diet had an ADG of 0.27 ± 0.19 kg/day.

Plasma urea nitrogen concentrations

There was an overall significant effect of treatment ($P < 0.0001$) and time ($P = 0.0006$) on plasma urea nitrogen concentraions, but the interaction was not significant ($P > 0.05$) (Table 5.4). The timothy-based diets resulted in significantly lower plasma urea nitrogen concentrations when compared to the alfalfa-based diets. Post-feeding plasma

urea nitrogen concentrations were significantly greater than pre-feeding concentrations. To further characterize the effect of treatment on plasma urea nitrogen concentrations, the effect of forage type and level of protein in the ration balancer, was analyzed as a 2x2 factorial design for each of the both time points (i.e. pre and post feeding).

For the pre-feeding samples, there was a significant effect of forage type on plasma urea nitrogen concentrations, where horses consuming alfalfa had greater levels than horses consuming timothy ($P = 0.0001$; Table 5.5). There was a significant effect of the protein level of the ration balancer on plasma urea nitrogen concentrations, where horses consuming the high protein balancer had greater concentrations than horses consuming the low protein balancer ($P < 0.0001$; Table 5.5). There was a significant effect of the interaction of forage type and protein level on plasma urea nitrogen concentration ($P = 0.0009$; Table 5.5). Within the two timothy forage diets, horses consuming the high protein ration balancer had greater plasma urea nitrogen concentrations when compared to horses consuming the low protein ration balancer, but plasma urea nitrogen concentrations in horses receiving alfalfa were not dependent on the protein intake of the ration balancer.

For the post-feeding samples, there was a significant effect of forage type on plasma urea nitrogen concentrations, where horses consuming alfalfa had greater levels than horses consuming timothy ($P < 0.0001$; Table 5.6). There was a significant effect of the protein level of the ration balancer on plasma urea nitrogen concentrations, where horses consuming the high protein balancer had greater concentrations than horses consuming the low protein balancer ($P < 0.0001$; Table 5.6). There was a significant

effect of the interaction of forage type and ration balancer protein level on plasma urea nitrogen concentration (P = 0.002) (Table 5.6). Within the two timothy forage diets, horses consuming the high protein ration balancer had greater plasma urea nitrogen concentrations when compared to horses consuming the low protein ration balancer, but when horses received alfalfa, there was no effect of ration balancer protein content on plasma urea nitrogen.

Plasma indispensable amino acid concentrations

Treatment significantly affected the branched chain amino acid (BCAA) concentrations, isoleucine ($P = 0.0001$), leucine ($P < 0.0001$) and valine ($P < 0.0001$), where horses consuming alfalfa treatments had greater levels of BCAA on average than horses consuming timothy treatments (Table 5.4). There was a significant effect of time on all indispensable amino acids studied, where the 90 min post-feeding concentrations were greater than the pre-feeding concentrations ($P < 0.0001$; Table 5.4). A significant treatment by time interaction was seen for plasma isoleucine ($P = 0.05$) and lysine ($P = 0.02$) concentrations (Table 5.4). An effect of sample time was seen for plasma isoleucine concentrations, where concentrations were lowest when horses consumed timothy and the low protein pellet. Plasma lysine concentrations were lowest when horses consumed timothy and the low protein pellet, compared to all other treatments. To better understand the treatment effects, the effect of forage type and protein level was analyzed for each time point separately.

Pre-feeding plasma isoleucine ($P = 0.0003$), leucine ($P < 0.0001$), and valine ($P < 0.0001$) concentrations were affected by forage type, with alfalfa diets resulting in higher

plasma concentrations than the timothy diets (Table 5.5). Pre-feeding plasma lysine (P = 0.02), threonine (P = 0.002), tryptophan (P = 0.05), and valine (P = 0.02) concentrations were affected by the protein level of the ration balancer, with higher concentrations seen when horses received the high protein versus the low protein ration balancer (Table 5.5). There was a significant effect of the forage by protein level interaction on plasma isoleucine concentrations (P = 0.003) (Table 5.5), where plasma isoleucine concentrations in horses receiving the timothy and low protein ration balancer were lower than those in those horses receiving the timothy and high protein ration balancer, but there was no effect of ration balancer protein content on plasma isoleucine concentrations in horses receiving alfalfa.

In the post-feeding samples, there was a significant effect of forage type on plasma isoleucine (P = 0.01), leucine (P = 0.0008), and valine (P < 0.0001) concentrations, again with alfalfa diets resulting in higher plasma concentrations than the timothy diets (Table 5.6). Post-feeding plasma isoleucine (P = 0.001), leucine (P = 0.05), lysine (P = 0.01), and valine (P = 0.004) concentrations were affected by the protein level of the ration balancer, with horses receiving the high protein ration balancer having greater plasma concentrations of these amino acids than those receiving the low protein ration balancer (Table 5.6). There were no significant effects of the forage type by ration balancer protein level interaction for any of the post-feeding plasma indispensable amino acid concentrations (P > 0.05; Table 5.6).

Plasma dispensable amino acid concentrations

There was a significant effect of treatment on plasma concentrations of alanine ($P = 0.05$), arginine ($P = 0.05$), asparagine ($P = 0.004$), glutamate ($P < 0.0001$) and serine ($P < 0.0001$; Table 5.4). Of the plasma amino acids affected by treatment, plasma concentrations were greater in timothy-based diets, with the exception of arginine and asparagine, where plasma concentrations were lower in the alfalfa-based diets. There was a significant effect of time on the concentrations of all the dispensable plasma amino acids studied ($P < 0.0001$), except for glycine ($P = 0.3381$; Table 5.4). Of the plasma amino acids affected by time, plasma concentrations were greater post-feeding, with the exception of citrulline, where plasma concentrations were lower post-feeding. There was a significant interaction of treatment by time for plasma arginine ($P = 0.009$) and asparagine ($P = 0.01$) concentrations (Table 5.4). When horses consumed the high protein ration balancer, plasma concentrations of arginine were higher when compared to horses consuming the low protein pellet. When horses consumed alfalfa-based diets, plasma concentrations of asparagine increased when compared to horses consuming the timothy-based diets. To better understand the treatment effects, the effect of forage type and protein level was analyzed for each time point separately.

Pre-feeding concentrations of plasma alanine ($P = 0.02$), asparagine ($P = 0.05$), aspartate ($P = 0.02$), citrulline ($P = 0.008$), glutamate ($P < 0.0001$), and glutamine ($P = 0.05$) were significantly affected by forage type (Table 5.5). Of the plasma amino acids affected by forage, plasma concentrations were greater in horses consuming the alfalfa-based diets, with the exceptions of alanine, citrulline, glutamate and glutamine where plasma concentrations were lower in horses consuming the timothy-based diets. Pre-feeding plasma alanine ($P = 0.005$), asparagine ($P = 0.0002$), glutamate ($P = 0.02$),

glutamine ($P = 0.001$), glycine ($P = 0.02$), proline ($P = 0.002$) and serine ($P < 0.0001$), concentrations were affected by the protein level of the ration balancer (Table 5.5). Of the plasma amino acids affected by protein level of the ration balancer, plasma concentrations were greater when horses consumed the low protein pellets when compared to horses consuming the high protein pellets. There was a significant effect of the interaction of forage by protein level on plasma glutamate ($P = 0.02$) and serine ($P = 0.05$) concentrations (Table 5.5). For both glutamate and serine, horses receiving timothy and the high protein ration balancer had greater plasma concentrations compared to those receiving timothy and the low protein pellet; however, this effect was not seen when horses received alfalfa.

In the post-feeding samples, there was a significant effect of forage type on plasma alanine ($P = 0.02$), asparagine ($P < 0.0001$), aspartate ($P = 0.002$), glutamate ($P < 0.0001$), and serine ($P = 0.05$) concentrations (Table 5.6). Of the plasma amino acids affected by forage, plasma concentrations were greater in the alfalfa-based diets, with the exceptions of alanine, aspartate, glutamate and serine where plasma concentrations were lower in the timothy-based diets. Post-feeding plasma arginine ($P = 0.005$) and serine ($P < 0.0001$) concentrations were affected by the protein level of the ration balancer (Table 5.6). Plasma arginine concentrations were greater when horses consumed the high protein pellets when compared to horses consuming the low protein pellets. Plasma serine concentrations were greater when horses consumed the low protein pellets when compared to horses consuming the high protein pellets. There were significant effects of the forage type by ration balancer protein level interaction for the post feeding plasma concentrations of arginine ($P = 0.04$), glutamate ($P = 0.03$) and serine ($P = 0.0008$)

(Table 5.6). Plasma arginine concentrations were lower and the plasma glutamate and serine concentrations were higher when horses receiving the timothy forage received the high protein versus low protein ration balancer; however, this effect was not seen when horses received the alfalfa forage.

Whole-body phenylalanine kinetics

There was not a significant overall effect of treatment on total CO_2 production ($P > 0.05$; Table 5.7). There was a significant overall effect of treatment on phenylalanine flux ($P = 0.001$; Table 5.7), where flux rates of the horses that consumed the two alfalfa treatments were significantly higher than in horses consuming the timothy diets. The difference between phenylalanine flux and oxidation represents non-oxidative phenylalanine disposal, which is an estimate of phenylalanine use for protein synthesis. Phenylalanine oxidation ($P = 0.004$) and protein synthesis ($P = 0.009$) followed the same patterns as phenylalanine flux, with a significant effect of treatment on these parameters (Table 5.7). Horses receiving the alfalfa diets had greater rates of oxidation and phenylalanine use for protein synthesis than horses receiving the timothy diets. Horses consuming alfalfa and the high protein balancer had the greatest levels of oxidation and phenylalanine for protein synthesis, but this treatment's effects were not different compared to horses consuming alfalfa and the low protein balancer. When horses consumed either of the timothy treatments, rates of oxidation and phenylalanine for protein synthesis were lowest. Generally, as the concentration of crude protein in the diet decreased, so did phenylalanine oxidation and protein synthesis.

In order to discern which treatment factors were responsible for the significant differences across treatments, data were analyzed with forage type and protein level of the ration balancer as the two variables in a factorial design. There was an effect of forage type on the rate of phenylalanine flux ($P = 0.0003$; Table 5.8), phenylalanine oxidation ($P = 0.0009$; Table 5.8) and phenylalanine use for protein synthesis ($P = 0.002$; Table 5.8), but only phenylalanine flux was affected by ration balancer protein content ($P = 0.046$; Table 5.8). There were greater rates of phenylalanine flux, oxidation and synthesis resulting from the alfalfa diets when compared to timothy diets. There was an effect of level of ration balancer protein content, where phenylalanine flux was greatest in horses receiving the high protein ration balancer. There was no effect of the forage by ration balancer protein level interaction ($P > 0.05$; Table 5.8). There was no significant effect of either forage type, protein level, or their interaction on total CO_2 production ($P > 0.05$; Table 5.8).

Discussion

This study was the first to use isotope kinetics to evaluate the effects of forages supplemented with either a high or low protein ration balancer on measures of whole-body protein metabolism in growing horses. The majority of the differences seen in this study were in response to the different forage types, rather than as a result of the ration balancer protein content. This suggests that forage type was the major determinant of whole-body protein status in the growing yearlings used in the present study. Within each forage type, phenylalanine kinetics were not influenced by ration balancer protein content, suggesting that no amino acids were limiting with either forage type. However, horses receiving the alfalfa forage treatments, which provided more crude protein and

indispensable amino acids than timothy, did have greater rates of whole-body protein synthesis, independent of ration balancer protein content, when compared to the timothy forage treatments. When horses consumed the timothy and low protein pellet, plasma concentrations of urea nitrogen and branched chain amino acids (BCAA) were lowest. Reduced plasma urea nitrogen concentrations in horses consuming this treatment diet is likely indicative of increased rates of endogenous recycling of indispensable amino acids, which utilize BCAA for transamination reactions. Plasma concentrations of alanine and glutamine, known nitrogen-carriers, were greatest when horses consumed the timothy and low protein pellet.

The NRC estimates that horses willingly eat 2-3% of their BW each day as forage on an as fed basis (NRC, 2007). The yearlings in the present study were offered 2-2.5% of their BW as forage on non-sampling days and consumed an average of 1.84% of their BW as forage on an as-fed basis. On average, only about 20% of the forage provided each day was refused, and there were no refusals of ration balancer. Approximately 75% of the horses' diets were comprised of forage and the remaining 25% from ration balancer.

In previous studies looking at protein metabolism in yearlings, a 60:40 grain:hay ratio (Antilley et al., 2007; Ott et al., 1981) was used, so by comparison, diets in this study were primarily forage-based. When compiling responses from 58 farm surveys representing 1,987 young horses, it was found that only 9% of farms fed more grain than hay (Gibbs and Cohen, 2001). Of the 28% of farms surveyed with no access to pasture, the majority (70%) fed yearlings equal or more parts of forage to grain in a ratio of either

50:50, 40:60 or 30:70 grain:hay. Only 20% of respondents fed more grain than hay (20:80 or 10:90), signifying that the majority of people feeding young horses from this survey rely on a predominantly forage-based diet (Gibbs and Cohen, 2001). Although the treatments in the present study were right at the higher end of forage inclusion, the results from this study are very much industry relevant.

When amino acids are degraded by the body, the amino moiety is eventually converted to urea via the hepatic urea cycle and the carbon backbones are converted to CO_2 and disposed of via exhalation. For this reason, plasma urea nitrogen concentrations are often used as a measure of amino acid degradation. There was a significant interaction between forage type and ration balancer protein level once data was split into the pre-feeding and post-feeding sample times, where plasma urea nitrogen concentrations were higher when horses received timothy and high protein pellets compared to the low protein pellet, but there was no effect of the protein level within in the alfalfa-based treatments. However, the reason for the lack of response of the alfalfa treatments to ration balancer protein content is unknown. When horses were fed the timothy and low protein pellet, plasma urea nitrogen concentrations were the lowest. This is congruent with the lower protein content of the timothy hay and is supported by the lower amino acid intake within the timothy and low protein group. Conversely, when horses were fed the alfalfa and high protein pellet, plasma urea nitrogen concentrations were highest, again reflecting the elevated protein and amino acid intake for this treatment. Excess amino acids cannot be stored and must be catabolized, so when amino acid intake exceeds requirements, plasma urea nitrogen concentrations increase due to increased rates of amino acid catabolism. A previous study reported higher plasma urea

nitrogen concentrations in yearlings fed a 20% CP concentrate compared to those fed a 14% CP concentrate (Schryver et al., 1987), and is consistent with the literature from swine where higher dietary protein intake results in an increase in plasma urea nitrogen concentrations (Zervas and Zijlstra, 2002). Yearling horses allowed ad libitum access to an 18% CP pasture and supplemented with an unspecified amount of either a 14% CP concentrate, or a 9% CP concentrate fortified with lysine and threonine, did not have different plasma urea nitrogen concentrations (Staniar et al., 2001). Average plasma urea nitrogen concentrations for both treatment groups were 6.7 mmol/L and in the present study the range of plasma urea nitrogen concentrations, across all treatments and time points, was 3.2 to 7.1 mmol/L (Staniar et al., 2001). According to Cornell University's Veterinary Diagnostics Lab, the normal range of reference of serum urea nitrogen concentrations in mature horses is 3.57-7.85 mmol/L. The lowest concentrations of plasma urea nitrogen were seen both pre- and post-feeding in horses receiving the timothy and low protein pellet, and were below the average range of reference. It is possible that growing horses may have a wider range of reference for plasma urea nitrogen concentrations, but no evidence of this could be found in the consulted literature.

It has previously been shown in swine that if a limiting dietary amino acid, such as lysine, is added to the test diets, there is a decrease in plasma urea nitrogen concentrations compared to diets limiting in lysine (Coma et al., 1995). In the current study, as the protein content of the total diets increased, so did the plasma urea nitrogen concentrations, indicating that amino acid requirements were met with either forage in combination with a low protein ration balancer. As plasma urea nitrogen concentrations increase, so do rates of degradation, indicating that extra protein intake coming from high

protein diets provided a surplus of amino acids needed by these horses. Because the addition of the high protein ration balancer increased the intake of all amino acids and not just a selected potentially limiting amino acid, it is not possible to conclude from plasma urea nitrogen data alone whether or not there were limiting amino acids when the low protein ration balancer was used.

Plasma concentrations of the indispensable amino acids are influenced by dietary intake, whole-body protein turnover, and the rate of amino acid degradation. It is important to note that although plasma amino acids concentrations give us a snapshot of the whole-body free amino acid pools and can provide indications for changes in whole-body metabolism, they do not allow us to make definitive conclusions regarding the mechanisms responsible for the differences in plasma concentrations. Despite large differences in amino acid intakes between treatment groups, there were only a few amino acids that were affected by forage and protein level, specifically the BCAA. Previously it has been shown that the plasma concentrations of other amino acids, specifically lysine and threonine, are responsive to dietary inclusion levels (Mastellar et al., 2016a; Mastellar et al., 2016b; Mok et al., 2018), so the lack of plasma amino acid responses in this study were surprising.

On blood sampling days horses were fasted for ~15 hours prior to their first blood sample, then fed 0.35% of their body weight of their ration balancer allocation (half of their total daily concentrate intake) and only 1kg of hay once they had finished their ration balancer. The mean retention time of alfalfa hay in the equine digestive tract has been found to be around 20 hours (Pearson et al., 2001) and between 18-23 hours for timothy hay (Uden et at., 1982), so it is likely that forage from the previous afternoon's

meal was still retained in the large intestine for the pre-feeding sample. Additionally, the concentrate made up a small portion of the horse's total diet, and therefore the differences seen may not have been large enough to be statistically detected. As expected, plasma amino acids concentrations for all amino acids studied increased after feeding, which reflects that within the 90-minute period a portion of the meal had been absorbed from the gastrointestinal tract, which was similar to previous reports. (Tanner et al., 2014; Mastellar et al., 2016a; Mastellar et al., 2016b; Mok et al., 2018). Only minimal amounts of forage consumed was likely digested in that 90 min period, as it would be digested primarily in the large intestine (Gibbs et al., 1988). In the large intestine, protein is digested microbially and the products available for absorption are mainly ammonia and not individual amino acids (Meyer, 1983). Conversely, concentrates are primarily digested in the foregut and has been proven to demonstrate a response to a meal after 90 minutes (Gustavsson et al., 2010; Mastellar et al., 2016a; Mastellar et al., 2016b; Mok et al., 2018; Tanner et al., 2014; Wagner et al., 2013). Therefore, the observed increase in plasma amino acid concentrations at 90 min is likely caused by the protein provided by the ration balancer.

Mature horses fed alfalfa with a comparable CP to the alfalfa fed in the present study had an apparent total tract nitrogen digestibility of 74% (Cuddeford et al., 1992). Whereas, mature horses fed timothy with a comparable CP to the timothy fed in the present study had an apparent total tract digestibility of 65% (Darlington et al., 1968). It has been reported that that the majority of the digestion of forage protein occurs post-cecally (Gibbs et al., 1988). Differences in the digestibility of feedstuff, as well as the site of digestion, greatly impact the extent to which dietary amino acids are absorbed.

Woodward et al. investigated the abundances of cationic and neutral amino acid transporters in different segments of the gastrointestinal tract (Woodward et al., 2010). Results from this study indicate that in the hindgut of the horse, there is a presence of cationic and neutral amino acid transporters. It has been reported in ponies that free lysine is able to be transported across the brush border membrane in the colon and, from the transporters studied, there is a greater capacity and affinity for lysine transport in the colon compared to the jejunum (Woodward et al., 2012). However, more research is required to determine to what extent other amino acids are able to be absorbed and utilized from the large intestine. Therefore, plasma concentrations at 90 min post-feeding might not a good representation of amino acid intake from forage, but rather the concentrate meal.

An interesting finding in the present study was that the group of amino acids most affected by both forages and ration balancer types were the branched chain amino acids (BCAA). The observed differences in plasma BCAA concentrations, both pre-feeding and post-feeding, were reflective of differences in BCAA intake between forages and between ration balancers. Recall that on average, the mean retention time for similar forages used in this study was between 18-23h, but that horses were fasted for only 15 hours prior to baseline blood samples, so it is possible that forage from the previous afternoon's meal was still retained in the large intestine for the pre-feeding sample, and could have also influenced the 90-minute post-feeding sample as well.

When horses consumed alfalfa and the high protein ration balancer, plasma levels of BCAA were higher when compared to their lower protein counterparts. BCAA help to regulate body protein synthesis and represent the major nutrition source for glutamine

and alanine synthesis in muscle (Ruderman et al., 1974). One way in which BCAA are unique compared to other amino acids is the fact that they are primarily oxidized by the skeletal muscle, as compared to the other indispensable amino acids which are primarily oxidized in the liver (DeSantiago et al., 1998; Dillon, 2013; Cole, 2015). This difference in the primary site of oxidation could be a potential explanation for why the plasma BCAA concentrations were more influenced by treatment than any of the other indispensable amino acids. Differences in plasma BCAA concentrations between treatments could be attributed to differences between dietary BCAA intake. The increased levels of BCAA in horses consuming alfalfa and the high protein pellet may have promoted an increase in whole-body protein synthesis. This is supported by the fact that phenylalanine use for protein synthesis was highest in the treatments that resulted in the highest plasma BCAA concentrations. Leucine is not only a substrate for protein synthesis, but in other species it has been shown to promote protein synthesis and inhibit protein degradation through the mTOR signaling pathway (Mordier et al., 2000; Yoshiharu et al., 2006; Drummond and Rasmussen 2008; Chen et al., 2011). This could be a factor that partially explains why there were elevated rates of protein synthesis seen in horses receiving the alfalfa diets.

Despite the fact that the timothy hay and the low protein ration balancer contained less alanine, glutamate, glutamine, and glycine than the alfalfa hay and high protein ration balancer, plasma concentrations of these amino acids were consistently highest in the timothy and low protein ration balancer treatment. Alanine, glutamate, glutamine, and glycine are known nitrogen carriers that readily donate and receive amino groups, often from BCAA and their ketoacid counterparts, through different transamination reactions

(Brosnan, 2003). Elevated concentrations of known nitrogen carriers are indicative of limited protein synthesis, resulting in the need to remove amino acids that are not able to be used for protein synthesis for metabolism to urea and subsequent excretion. This 'recycling' of nitrogen carriers through transamination is important not only to clear blood levels of excess ammonia in times of amino acid excess, but also for amine groups and carbon backbones transportation to other tissues for *de novo* protein synthesis in the body (Christensen, 1990). Although all amino acid requirements were met in the timothy and high protein dietary treatment, lysine was deficient in the timothy and low protein diet. It is possible that due to the deficiency of lysine in this treatment, whole-body protein synthesis was either not optimally using amino acids, or that there was an increase in protein degradation in the timothy treatments, resulting in more amino acids destined for catabolism. However, this claim is not supported by the plasma urea nitrogen concentrations of horses fed timothy and the low protein pellet. Therefore, it is unknown why this treatment resulted in the highest plasma concentrations of these amino acids.

Stable isotope infusion methodologies allow us to model the whole-body movement of amino acids, in this case phenylalanine, into and out of the free amino acid pool and make estimates of rates of phenylalanine oxidation, use for protein synthesis and release from protein degradation. Phenylalanine kinetics can be influenced by many factors such as the horse's age or diet (Mastro et al, 2014; Tanner et al. 2014; Latham, 2016). In the present study there was an effect of treatment on phenylalanine flux, oxidation and use for protein synthesis, which when analyzed further were primarily due to differences in forage types. Horses receiving the alfalfa-based diets had greater rates of phenylalanine flux, oxidation and use for protein synthesis when compared to horses

receiving the timothy-based diets. Phenylalanine flux is the rate of phenylalanine entry and exit into the free amino acid pool and is influenced largely by dietary phenylalanine intake. Horses consumed greater amounts of phenylalanine when they received the alfalfa treatment, which explains the greater phenylalanine flux for these treatments. Despite differences in phenylalanine intake between the treatment groups, there was no effect of forage type on plasma phenylalanine concentrations, either pre- or post-feeding, indicating that rates of phenylalanine entering the body amino acid pool were equal to the rate of amino acids exiting the body amino acid pool. When amino acids are consumed, they have two primary metabolic fates: they may be used to support protein synthesis or they are metabolized, with the carbon backbone oxidized to carbon dioxide. The elevated rate of phenylalanine oxidation in horses receiving the alfalfa treatments indicates that a portion of the elevated phenylalanine flux was metabolized through the oxidative pathways, with the remainder of the elevation being accounted for by the elevated use of phenylalanine for protein synthesis.

Although it is assumed that non-oxidative phenylalanine disposal is indicative of whole-body protein synthesis, because there is not a good estimate of the requirement for tyrosine in horses and because tyrosine and phenylalanine intakes differed between diets, the increase in non-oxidative phenylalanine disposal could be due to increased phenylalanine conversion to tyrosine. However, given that the treatments with the greatest non-oxidative phenylalanine disposal were also the treatments providing the highest amount of dietary phenylalanine and tyrosine, this seems unlikely. Differences in whole body-protein synthesis between treatments may be even greater than what would

have been measured if the conversion of phenylalanine to tyrosine was able to be directly measured in all of the treatments.

The balance between the rate of phenylalanine use for protein synthesis and release from protein degradation is indicative of the whole-body rate of protein accretion. In mature horses, rates of phenylalanine use for protein synthesis are equal to or lower than rates of phenylalanine release from protein degradation (Urschel et al., 2012; Wagner et al., 2013; Mastro et al, 2014; Tanner et al., 2014). In growing horses, however, rates of protein synthesis would be expected to exceed rates of protein degradation, resulting in the accretion of body protein (Tanner et al., 2014; Mastellar et al., 2016a; Mastellar et al., 2016b). Although it was found that phenylalanine used for protein synthesis was greatest in horses receiving the two alfalfa treatments, estimates for dietary phenylalanine digestibility or splanchnic phenylalanine extraction are not currently available in the literature. Therefore, the rate of phenylalanine release from protein degradation could not be determined and thus it cannot be definitively concluded whether or not these elevated rates of protein synthesis resulted in greater rates of whole-body protein accretion in the horses receiving the alfalfa treatments. However, the elevated rates of protein synthesis when horses received the alfalfa treatments is in line with the numerically greater rate of average daily gain in yearlings consuming the alfalfa-based diets versus timothy-based diets.

Numerically, ADG was greatest for the alfalfa and low protein ration balancer treatment, followed by the alfalfa and high protein ration balancer treatment, the timothy and high protein ration balancer treatment and finally the timothy and low protein ration

balancer treatment. The effect of forage on ADG was very close to reaching significance ($P = 0.056$), which agrees with the protein synthesis data. When this experiment was designed, the primary endpoint on which the power calculation was based was the phenylalanine kinetics measures. From previous research in our lab, 5-6 horses per treatment is sufficient to measure differences between treatments in whole-body phenylalanine kinetics parameters (Urschel et al., 2012; Tanner et al., 2014); however, this number was unlikely to provide sufficient power to detect differences in growth rate. Additionally, isotope kinetic methodologies have the advantage that they are able to detect differences in whole-body protein metabolism with minimal time needed for adaptation to treatment diets, with adaptation times of less than a week commonly used in studies in swine and humans (Zello et al., 1990; Pencharz and Ball, 2003; Myrie et al., 2008; Elango et al., 2009; Myrie et al., 2014). Previous studies in yearlings using growth as an endpoint typically used a larger number of subjects, ranging from 68 to 1992 horses, and for a longer duration of time, ranging from 217-674 days (Hintz et al., 1979; Ott et al., 1979; Thompson, 1995). The lack of a significant effect of forage or ration balancer protein level on ADG in this study is likely a function of high inter-horse variability combined with the short sampling periods and low sample numbers.

Horses receiving the timothy and low protein ration balancer diet had overall lower than normal rates of ADG. In a different study, the ADG of yearlings were not altered by crude protein intake (Staniar et al, 2001). ADGs were 0.71 kg/day when horses consumed either a 14% CP control diet and were 0.74, kg/day when horses consumed a 9% CP diet fortified with lysine and threonine (Staniar et al, 2001). According to the NRC, the normal range of ADG for thoroughbreds aged 11 months is 0.4 to 0.5 kg/day

and for horses aged 13 to 15 months, 0.4 to 0.7 kg/day (Asai, 2000, NRC 2007). The ADGs from the present study ranged from 0.27 to 0.62, with horses consuming alfalfa and the low protein ration balancer having the numerically greatest ADG and horses consuming timothy and the low protein ration balancer having the lowest ADG. ADG has been seen to wane in winter months, which is likely a function of horses using energy to shiver when external temperatures fall below the horses' thermal-neutral zone (Pagan et al., 1996; Asai, 2000; Staniar et al., 2004; NRC 2007). The first three weeks of the present study was conducted during winter; however, all four treatments were equally represented during this timeframe and should not be the cause for any differences between treatments. The energy contents of diets were similar between treatments and well exceeded the NRC recommendations (NRC, 2007). The only treatment where dietary lysine was below the estimated requirement was the timothy and low protein ration balancer diet and it is possible that lysine was limiting in this diet. However, if lysine truly was limiting, it would have been expected to see an increase in protein synthesis with the timothy and high protein ration balancer diet in comparison, which was not observed. It is possible that the NRC's estimate of lysine overestimates the requirement for growing horses.

Conclusion

In conclusion, the results from the current study suggest that yearling horses receiving the alfalfa-based diets had better growth and greater rates of protein synthesis when compared to horses receiving the timothy-based diets. The addition of a ration balancer, either low or high in protein, did not seem to influence measures of whole-body

protein metabolism in yearling horses. This implies that the provision of both a good quality legume or grass hay, along with a small amount of a low protein ration balancer, is likely able to meet amino acid requirements for these horses. One of the limitations of this study was that with respect to ADG, the duration of study periods and the number of horses may not have been sufficient to see differences in growth between treatments; however, this was not the endpoint of this study. Isotope infusion techniques allowed for measures of whole-body phenylalanine kinetics, which provided support that greater levels of protein synthesis occurred in this study when horses consumed the alfalfa-based diets. It was hypothesized that the dietary treatment allocations in this study would influence measures of whole-body protein synthesis and growth in yearling horses. Although no differences were seen with respect to growth, measures of whole-body protein synthesis did indicate that alfalfa-based diets supported increased protein synthesis in yearling horses. Future research should focus on identifying other limiting amino acids of the growing horse and determining why the timothy-based diets resulted in lower rates of whole-body protein synthesis than the alfalfa-based treatments. From this study it can be concluded that yearling horses can achieve normal growth and greater rates of protein synthesis when fed alfalfa-based diets, particularly when compared to a timothy-based diets supplemented with a low protein ration balancer.

Tables

Table 5.1 Nutrient composition of each component of treatment diet, as-fed basis.

	Low Protein Ration Balancer	High Protein Ration Balancer	Alfalfa Hay	Timothy Hay
Overall nutrient composition[1]				
Dry Matter, %	87.63 ± 1.45	88.03 ± 0.25	90.97 ± 0.32	90.6 ± 0.70
DE, Mcal/kg	3.10 ± 0.10	2.90 ± 0.07	2.06 ± 0.05	1.98 ± 0.06
Crude protein, %	8.50 ± 0.17	30.47 ± 0.55	17.53 ± 0.55	10.43 ± 0.12
Acid Detergent Fiber, %	8.80 ± 0.20	11.40 ± 3.29	35.63 ± 0.65	34.90 ± 0.87
Neutral Detergent Fiber, %	14.77 ± 1.82	15.43 ± 1.81	42.27 ± 1.32	53.13 ± 1.55
Water Soluble Carbohydrates, %	6.80 ± 0.44	12.17 ± 0.15	6.77 ± 0.74	12.70 ± 0.30
Ethanol Soluble Carbohydrates, %	5.27 ± 0.23	9.70 ± 0.75	4.23 ± 0.42	6.23 ± 0.49
Starch, %	35.53 ± 1.55	2.33 ± 0.21	0.40 ± 0.10	0.43 ± 0.12
Calcium, %	3.85 ± 0.17	3.90 ± 0.09	1.29 ± 0.08	0.49 ± 0.04
Phosphorus, %	2.00 ± 0.08	1.99 ± 0.13	0.29 ± 0.00	0.24 ± 0.01
Magnesium, %	0.32 ± 0.02	0.38 ± 0.01	0.34 ± 0.01	0.22 ± 0.01
Potassium, %	0.71 ± 0.01	1.58 ± 0.02	2.12 ± 0.01	1.57 ± 0.04
Sodium, %	0.99 ± 0.03	0.48 ± 0.04	0.08 ± 0.00	0.02 ± 0.00
Iron, mg/kg	683 ± 134.37	596 ± 34.96	149± 16.29	179 ± 29.14
Zinc, mg/kg	435 ± 23.07	384 ± 9.07	16.00 ± 0.00	32.67 ± 1.15
Copper, mg/kg	151 ± 24.01	141 + 16.82	7.00 ± 0.00	7.00 ± 1.00
Manganese, mg/kg	302 ± 20.84	272 ± 12.50	26.33 ± 2.08	63.67 ± 3.79
Molybdenum, mg/kg	1.10 ± 0.17	5.10 ± 0.17	1.77 ± 0.06	0.73 ± 0.06
Amino acid composition, %[2]				
Alanine	0.28 ± 0.03	1.99 ± 0.13	1.44 ± 0.17	0.57 ± 0.19
Arginine	0.79 ± 0.04	3.86 ± 0.18	1.04 ± 0.12	0.76 ± 0.13
Aspartate and Asparagine	0.23 ± 0.02	1.13 ± 0.04	0.50 ± 0.02	0.31 ± 0.03
Glutamate and Glutamine	0.19 ± 0.01	0.81 ± 0.03	0.40 ± 0.03	0.28 ± 0.003
Glycine	0.10 ± 0.02	0.63 ± 0.05	0.22 ± 0.05	0.14 ± 0.01
Histidine	0.31 ± 0.01	1.70 ± 0.02	0.47 ± 0.07	0.33 ± 0.01
Isoleucine	0.18 ± 0.01	0.83 ± 0.02	0.42 ± 0.04	0.28 ± 0.01
Leucine	0.29 ± 0.01	0.88 ± 0.01	0.52 ± 0.03	0.37 ± 0.05
Lysine	0.35 ± 0.02	1.08 ± 0.02	0.71 ± 0.02	0.53 ± 0.04
Methionine	0.17 ± 0.01	0.79 ± 0.03	0.30 ± 0.01	0.18 ± 0.02
Phenylalanine	0.25 ± 0.003	0.95 ± 0.02	0.52 ± 0.10	0.33 ± 0.03
Proline	0.17 ± 0.01	0.48 ± 0.01	0.23 ± 0.03	0.20 ± 0.01
Serine	0.21 ± 0.01	0.95 ± 0.02	0.43 ± 0.09	0.25 ± 0.03
Threonine	0.53 ± 0.01	1.79 ± 0.04	0.80 ± 0.07	0.52 ± 0.02
Tyrosine	2.60 ± 0.16	3.30 ± 0.08	1.74 ± 0.04	1.78 ± 0.05
Valine	0.27 ± 0.02	1.29 ± 0.02	0.59 ± 0.07	0.36 ± 0.03

[1]Analyzed by Equi-Analytical Laboratories
[2]Analyzed by HPLC in our lab

Table 5.2: Feed ingredient composition of the low protein ration balancer[1], DM basis.

Ingredient	% of diet
Ground corn	57.25%
Flaxseed	5.00%
Wheat bran	5.00%
Alfalfa meal	5.00%
Dried whey	3.75%
Yeast culture	1.25%
Molasses	6.25%
Soybean oil	2.25%
Calcium phosphate	9.65%
Calcium carbonate	2.00%
Salt	1.25%
McCauley TM premix	0.50%
McCauley Vitamin premix	0.50%
McCauley Bioplex blend	0.125
Selenium yeast premix	0.10%
Mold inhibitor	0.10%
Ethoxyquin	0.03%

[1]Concentrate formulated for study by McCauleys Bros, Versailles, KY

Table 5.3 Calculated average daily nutrient intakes of growing horses fed either alfalfa or timothy hay and a high or low protein ration balancer

	Feedstuff				NRC (2007) Recommendation[1]
	Alfalfa + High protein	Alfalfa + Low protein	Timothy + High protein	Timothy + Low protein	Total Daily Intake
Nutrient					
DE, Mcal/kg BW	0.066	0.067	0.064	0.065	0.062
Crude protein, g/kg BW	6.0	4.5	4.4	2.9	2.59
Amino acid intake, mg/kg BW					
Alanine	176	135	143	102	
Arginine	222	125	192	94	84.4
Aspartate and Asparagine	456	336	265	145	
Glutamate and Glutamine	499	284	437	223	
Glycine	145	101	118	75	
Histidine	93	55	75	38	64.4
Isoleucine	161	109	122	70	61.1
Leucine	301	213	240	152	118.8
Lysine	264	161	198	95	111.0
Methionine	84	63	78	56	30.0
Phenylalanine	220	149	170	98	66.6
Proline	232	181	192	141	
Serine	189	126	147	84	
Threonine	151	105	120	74	67.7
Tyrosine	121	78	95	52	
Valine	181	132	139	90	68.8

[1] Calculated based on the expected mature weight of 600kg of 12-month-old horses as determined by the NRC.
Results presented on an as-fed basis.
Forages were provided at 2% of bodyweight on an as-fed basis.
Ration balancers were provided at 0.7% of bodyweight on an as-fed basis.

Table 5.4: Main effects of treatment and time on plasma concentrations of urea nitrogen and amino acids in growing horses fed diets with either alfalfa or timothy combined with a high or low protein ration balancer.

	Treatment								*P*-values		
Metabolite	Alfalfa + High Protein		Alfalfa + Low Protein		Timothy + High Protein		Timothy + Low Protein		Treatment	Time	Treatment * Time
	Pre	Post	Pre	Post	Pre	Post	Pre	Post			
PUN, mmol/L	6.6 ± 0.4^{A}	7.1 ± 0.4^{a*}	5.8 ± 0.4^{A}	6.5 ± 0.4^{a*}	6.3 ± 0.4^{A}	6.4 ± 0.4^{a}	3.2 ± 0.4^{B}	3.4 ± 0.4^{b}	<0.0001	0.006	0.33
Indispensable Amino Acids, µmol/L											
Histidine	43 ± 3	71 ± 7*	46 ± 3	68 ± 7*	51 ± 3	79 ± 7*	47 ± 3	61 ± 7*	0.37	<0.0001	0.32
Isoleucine	65 ± 4^{AB}	94 ± 5^{a*}	73 ± 4^{A}	82 ± 6^{a}	62 ± 4^{B}	87 ± 5^{a*}	50 ± 4^{C}	59 ± 6^{b}	0.0001	<0.0001	0.05
Leucine	130 ± 6^{A}	178 ± 11^{a*}	133 ± 6^{A}	154 ± 12^{ab}	101 ± 6^{B}	133 ± 11^{bc*}	89 ± 6^{B}	112 ± 12^{c*}	<0.0001	<0.0001	0.29
Lysine	80 ± 13	194 ± 27^{ab*}	100 ± 13	153 ± 28^{ab*}	95 ± 13	223 ± 27^{a*}	106 ± 13	144 ± 28^{b}	0.55	<0.0001	0.02
Methionine	29 ± 1	36 ± 2*	31 ± 1	40 ± 3*	30 ± 1	42 ± 2*	33 ± 1	40 ± 3*	0.34	<0.0001	0.35
Phenylalanine	52 ± 3	70 ± 5*	55 ± 3	68 ± 5*	51 ± 3	68 ± 5*	51 ± 3	60 ± 5*	0.63	<0.0001	0.37
Threonine	70 ± 10	108 ± 13*	100 ± 10	116 ± 13	66 ± 10	104 ± 13*	87 ± 910	108 ± 13*	0.42	<0.0001	0.25
Tryptophan	52 ± 3	70 ± 5*	55 ± 3	68 ± 5*	51 ± 3	68 ± 5*	51 ± 3	60 ± 5	0.63	<0.0001	0.32
Valine	269 ± 9^{A}	331 ± 15^{a*}	256 ± 9^{A}	292 ± 15^{ab*}	205 ± 9^{B}	260 ± 15^{b*}	176 ± 9^{C}	206 ± 15^{c*}	<0.0001	<0.0001	0.18
Dispensable Amino Acids, µmol/L											
Alanine	144 ± 13^{B}	224 ± 23^{ab*}	160 ± 13^{B}	213 ± 25^{b*}	154 ± 13^{B}	258 ± 23^{ab*}	209 ± 13^{A}	283 ± 25^{a*}	0.05	<0.0001	0.37
Arginine	74 ± 7	138 ± 14^{a*}	86 ± 7	121 ± 14^{ab*}	75 ± 7	160 ± 14^{a*}	67 ± 7	94 ± 14^{b*}	0.05	<0.0001	0.009
Asparagine	50 ± 4	109 ± 7^{a*}	66 ± 4	114 ± 7^{a*}	44 ± 4	81 ± 7^{b*}	58 ± 4	86 ± 7^{ab*}	0.004	<0.0001	0.01
Aspartate	4.7 ± 0.6	7.5 ± 0.7*	5.2 ± 0.6	8.3 ± 0.7*	3.7 ± 0.6	6.4 ± 0.6*	3.8 ± 0.6	5.5 ± 0.7*	0.06	<0.0001	0.30
Citrulline	69 ± 5	65 ± 4	75 ± 5	64 ± 4*	63 ± 5	64 ± 4	57 ± 5	53 ± 4	0.11	0.03	0.33
Glutamate	26 ± 2^{C}	31 ± 2^{b*}	28 ± 2^{BC}	30 ± 2^{b*}	32 ± 2^{B}	35 ± 2^{b*}	39 ± 2^{A}	41 ± 2^{a}	<0.0001	<0.0001	0.44
Glutamine	434 ± 21	604 ± 25*	478 ± 21	586 ± 27*	452 ± 21	613 ± 25*	526 ± 21	653 ± 27*	0.0705	<0.0001	0.33
Glycine	571 ± 4	596 ± 7	615 ± 4	623 ± 7	570 ± 4	610 ± 7	637 ± 4	654 ± 7	0.3428	0.3381	0.85

Table 5.4 continued

Proline	106 ± 12	215 ± 16*	124 ± 12	211 ± 16*	108 ± 12	215 ± 16*	124 ± 12	196 ± 16*	0.9727	<0.0001	0.10
Serine	224 ± 14^{B}	279 ± 14^{b*}	272 ± 14^{A}	309 ± 14^{b*}	201 ± 14^{B}	267 ± 14^{b*}	304 ± 14^{A}	371 ± 14^{a*}	<0.0001	<0.0001	0.23
Tyrosine	57 ± 3	80 ± 6*	62 ± 3	78 ± 7*	56 ± 3	90 ± 6*	58 ± 3	70 ± 7	0.4681	<0.0001	0.07

Data are presented as least square means ± standard error of the mean
Abbreviations: PUN, plasma urea nitrogen
^{AB} indicates differences between the main effect of treatment pre-feeding
^{ab} indicates differences between the main effect of treatment post-feeding
* indicates significant main effect of time on the post-feeding sample that is different from the pre-feed sample within the same treatment
Alfalfa + High Protein, n = 8; Alfalfa + Low Protein, n = 7; Timothy + High Protein, n = 8; Timothy + Low Protein, n = 8

Table 5.5: Main effects of forage type and ration balancer protein level on pre-feeding plasma concentrations of urea nitrogen and amino acids in growing horses fed diets with either alfalfa or timothy combined with a high or low protein ration balancer.

	Alfalfa		Timothy		*P*-values		
Metabolite	High Protein	Low Protein	High Protein	Low Protein	Forage	Protein Level	Forage * Protein Level
PUN, mmol/L	6.6 ± 0.4	5.8 ± 0.4	6.3 ± 0.4^{Y}	3.2 ± 0.4^{Z*}	0.0001	<0.0001	0.0009
Indispensable Amino Acids, µmol/L							
Histidine	43 ± 3	47 ± 3	51 ± 3	47 ± 3	0.24	0.99	0.25
Isoleucine	65 ± 4	73 ± 4	62 ± 4^{Y}	50 ± 4^{Z*}	0.0003	0.53	0.003
Leucine	130 ± 6	133 ± 6	100 ± 6*	89 ± 6*	<0.0001	0.35	0.13
Lysine	79 ± 13^{B}	100 ± 13^{A}	95 ± 13	102 ± 13	0.13	0.02	0.27
Methionine	29 ± 1	31 ± 1	30 ± 1	33 ± 1	0.39	0.10	0.85
Phenylalanine	52 ± 3	55 ± 3	51 ± 3	51 ± 3	0.18	0.37	0.54
Threonine	70 ± 10^{B}	100 ± 10^{A}	66 ± 10^{X}	87 ± 10^{Y}	0.22	0.002	0.56
Tryptophan	44 ± 6	58 ± 6	54 ± 6	59 ± 6	0.21	0.05	0.32
Valine	269 ± 9	256 ± 9	205 ± 9^{Y*}	176 ± 9^{Z*}	<0.0001	0.02	0.39
Dispensable Amino Acids, µmol/L							
Alanine	144 ± 13	160 ± 13	154 ± 13^{Z}	209 ± 13^{Y*}	0.02	0.005	0.10
Arginine	74 ± 7	86 ± 7	75 ± 7	67 ± 7	0.08	0.71	0.07
Asparagine	50 ± 4^{B}	66 ± 4^{A}	44 ± 4^{Z}	58 ± 4^{Y}	0.05	0.0002	0.78
Aspartate	4.7 ± 0.6	5.2 ± 0.6	3.7 ± 0.6	3.8 ± 0.6	0.02	0.56	0.67
Citrulline	69 ± 5	75 ± 5	63 ± 5	57 ± 5*	0.008	0.99	0.15
Glutamate	26 ± 2	28 ± 2	32 ± 2^{Z*}	39 ± 2^{Y*}	<0.0001	0.02	0.02
Glutamine	434 ± 21^{B}	478 ± 21^{A}	452 ± 21^{Z}	526 ± 21^{Y}	0.05	0.001	0.33
Glycine	571 ± 31	615 ± 31	570 ± 31	637 ± 31	0.66	0.02	0.63
Proline	106 ± 12	124 ± 12	108 ± 12	124 ± 12	0.81	0.002	0.84

Table 5.5 Continued

Serine	224 ± 14	272 ± 14	201 ± 14^{Z}	304 ± 14^{Y}	0.76	<0.0001	0.05
Tyrosine	57 ± 3	62 ± 3	56 ± 3	58 ± 3	0.21	0.08	0.51

Data are presented as least square means ± standard error of the mean
Abbreviations: PUN, plasma urea nitrogen
^{AB} indicates differences between the main effect of protein level of ration balancer within the alfalfa forage diets
^{XY} indicates differences between the main effect of protein level of ration balancer within the timothy forage diets
* indicates significant main effect of forage on the timothy sample that is different from the alfalfa sample within the protein level
Alfalfa + High Protein, n = 8; Alfalfa + Low Protein, n = 7; Timothy + High Protein, n = 8; Timothy + Low Protein, n = 8

Table 5.6: Main effects of forage type or of ration balancer protein level on post-feeding plasma concentrations of urea nitrogen and amino acids in growing horses fed diets with either alfalfa or timothy combined with a high or low protein ration balancer.

	Alfalfa		Timothy		*P*-values		
Metabolite	High Protein	Low Protein	High Protein	Low Protein	Forage	Protein Level	Forage * Protein Level
PUN, mmol/L	7.1 ± 0.4	6.6 ± 0.4	6.4 ± 0.4^{Y}	3.4 ± 0.4^{Z*}	<0.0001	<0.0001	0.002
Indispensable Amino Acids, µmol/L							
Histidine	71 ± 7	72 ± 7	79 ± 7	60 ± 7	0.69	0.08	0.06
Isoleucine	94 ± 5	81 ± 6	87 ± 5^{Y}	59 ± 6^{Z*}	0.01	0.001	0.17
Leucine	178 ± 11	152 ± 12	133 ± 11*	112 ± 12*	0.0008	0.05	0.83
Lysine	194 ± 27	157 ± 29	223 ± 27^{Y}	142 ± 29^{Z}	0.77	0.01	0.34
Methionine	36 ± 2	40 ± 3	42 ± 2	40 ± 3	0.21	0.65	0.20
Phenylalanine	70 ± 5	69 ± 5	68 ± 5	60 ± 5	0.26	0.28	0.46
Threonine	108 ± 13	118 ± 14	104 ± 13	106 ± 14	0.41	0.54	0.70
Tryptophan	72 ± 5	73 ± 6	81 ± 5	70 ± 6	0.51	0.29	0.19
Valine	331 ± 15	289 ± 16	260 ± 15^{Y*}	205 ± 16^{Z*}	<0.0001	0.004	0.67
Dispensable Amino Acids, µmol/L							
Alanine	224 ± 24	220 ± 25	258 ± 24	287 ± 25	0.02	0.56	0.43
Arginine	138 ± 14	127 ± 15	160 ± 14^{Y}	95 ± 15^{Z}	0.70	0.005	0.04
Asparagine	109 ± 7	115 ± 8	81 ± 7	85 ± 8	<0.0001	0.38	0.93
Aspartate	7 ± 0.7	9 ± 0.7	6 ± 0.7	5 ± 0.7*	0.002	0.99	0.08
Citrulline	65 ± 4	64 ± 5	63 ± 4	54 ± 5	0.09	0.15	0.17
Glutamate	31 ± 2	31 ± 2	35 ± 2^{Z}	41 ± 2^{Y}	<0.0001	0.07	0.03
Glutamine	604 ± 25	594 ± 27	613 ± 25	657 ± 27	0.11	0.45	0.22
Glycine	596 ± 29	627 ± 30	610 ± 29	650 ± 30	0.35	0.09	0.81

Table 5.6 Continued

Proline	215 ± 16	216 ± 17	215 ± 16	192 ± 17	0.21	0.26	0.22
Serine	279 ± 14	307 ± 15	267 ± 14^{Z}	375 ± 15^{Y*}	0.05	<0.0001	0.008
Tyrosine	80 ± 6	80 ± 67	90 ± 6	69 ± 7	0.91	0.10	0.12

Data are presented as least square means ± standard error of the mean
Abbreviations: PUN, plasma urea nitrogen
^{XY} indicates differences between the main effect of protein level of ration balancer within the timothy forage diets
* indicates significant main effect of forage on the timothy sample that is different from the alfalfa sample within the protein level
Alfalfa + High Protein, n = 8; Alfalfa + Low Protein, n = 7; Timothy + High Protein, n = 8; Timothy + Low Protein, n = 8

Table 5.7: Effect of treatment on parameters of phenylalanine flux in growing horses fed diets with either alfalfa or timothy combined with a high or low protein ration balancer, μmol/(kg·h)

	Treatment				*P*-value
	Alfalfa High	Alfalfa Low	Timothy High	Timothy Low	Treatment
Phenylalanine flux[1]	75 ± 4^{A}	62 ± 4^{AB}	53 ± 4^{B}	49 ± 4^{B}	0.001
Phenylalanine oxidation[2]	38 ± 3^{A}	31 ± 2^{AB}	26 ± 3^{B}	23 ± 3^{B}	0.004
Phenylalanine for protein synthesis[3]	37 ± 2^{A}	31 ± 2^{AB}	27 ± 2^{B}	26 ± 2^{B}	0.009
CO_2 production[4]	17412 ± 1574	17860 ± 1363	16821 ± 1457	19311 ± 1457	0.67

[1] Flux = isotope infusion rate × [(isotope solution enrichment/ plateau plasma enrichment)-1]
[2] Oxidation = 100 × [(1/plateau plasma enrichment – 1/isotope solution enrichment) × rate of $^{13}CO_2$ release during phenylalanine infusion]
[3] Phenylalanine for protein synthesis = Non-oxidative Phenylalanine disposal = Flux – Oxidation
[4] Total carbon dioxide production = isotope infusion rate × [(isotope solution enrichment/ plateau breath enrichment)-1] × [0.0224 mL/μmol CO_2]
^{AB} indicates differences between the main effect of treatment
Alfalfa + High Protein, n = 8; Alfalfa + Low Protein, n = 8; Timothy + High Protein, n = 8; Timothy + Low Protein, n = 7

Table 5.8: Effect of forage type or of ration balancer protein level on parameters of phenylalanine flux in growing horses fed diets with either alfalfa or timothy combined with a high or low protein ration balancer, µmol/(kg·h)

	Treatment				*P*-value		
	Alfalfa High	Alfalfa Low	Timothy High	Timothy Low	Forage	Protein Level	Forage * Protein
Phenylalanine flux[1]	75 ± 4^{Aa}	62 ± 4^{Ab}	53 ± 4^{Ba}	49 ± 4^{Bb}	0.0003	0.046	0.3
Phenylalanine oxidation[2]	38 ± 3^{A}	31 ± 2^{A}	26 ± 3^{B}	23 ± 3^{B}	0.0009	0.06	0.4
Phenylalanine for protein synthesis[3]	37 ± 2^{A}	31 ± 2^{A}	27 ± 2^{B}	26 ± 2^{B}	0.002	0.1	0.3
CO_2 production[4]	17412 ± 1574	17860 ± 1363	16821 ± 1457	17412 ± 1574	0.8	0.3	0.5

[1] Flux = isotope infusion rate × [(isotope solution enrichment/ plateau plasma enrichment)-1]
[2] Oxidation = 100 × [(1/plateau plasma enrichment – 1/isotope solution enrichment) × rate of $^{13}CO_2$ release during phenylalanine infusion]
[3] Phenylalanine for protein synthesis = Non-oxidative Phenylalanine disposal = Flux – Oxidation
[4] Total carbon dioxide production = isotope infusion rate × [(isotope solution enrichment/ plateau breath enrichment)-1] × [0.0224 mL/µmol CO_2]
[AB] indicates differences between the main effect of forage
[ab] indicates differences between protein levels of ration balancers
Alfalfa + High Protein, n = 8; Alfalfa + Low Protein, n = 8; Timothy + High Protein, n = 8; Timothy + Low Protein, n = 7

Chapter 6: General discussions and future directions

It has been well established that forage-based diets are the backbone of the equine diet, and a good quality forage can meet the caloric and protein needs of sedentary mature horses. Despite this knowledge, horses are typically fed concentrates to provide additional energy or nutrients such as vitamins, minerals, or protein. However, supplementation of concentrates to forage diets can result in incidents of protein overfeeding, resulting in a necessary increase of nitrogen excretion, which ultimately ends up in the environment. As horses have a requirement for amino acids rather than protein, knowing the amino acid profile of the diet components is essential. The amino acids provided by the forage component of the diet depends on protein source and quality, which is influenced by different factors such as forage type, species, stage of maturity, and environmental factors. Typically, legume hays are higher in crude protein than grass hays and thus provide more of all the amino acids. Because the majority of horses' diets are typically provided as forage, amino acids provided from forage sources have a significant role in whole-body protein metabolism. Whole-body protein synthesis is determined by the profile of essential amino acids provided, and it is currently unknown if a forage only diet can provide correct ratios of limiting amino acids to horses. Because amino acids consumed above the most limiting amino acid are unable to be stored, requirements for amino acids that limit protein synthesis must be met to achieve optimum levels of protein synthesis. Thus, it is important to pair the amino acid provisions of forage with the amino acid requirements of the horse so that limiting amino acid requirements are met, but without overfeeding the other essential amino acids.

Despite the fact that protein is the largest non-water component comprising the body, little research regarding protein metabolism in horses in response to different forages has been published. These are the first studies to use isotope infusion methods, along with other criterion, to evaluate the effects of primarily forage-based diets supplemented with either amino acids or different ration balancers on measures of whole-body protein metabolism in mature and yearling horses, respectively. It was hypothesized that if a forage source was limiting in one or more indispensable amino acids, then protein quality would be improved by the addition of limiting amino acid supplements or ration balancers, resulting in greater rates of whole-body protein synthesis.

It was seen in the first study that mature horses at maintenance derived no benefit from amino acid supplementation. Although amino acid intake was higher when horses consumed alfalfa, compared to timothy, mature horses at maintenance did not benefit from amino acid supplementation with respect to measures of whole-body protein metabolism. The amount of oxidized phenylalanine and plasma urea nitrogen concentrations were greater when horses consumed the alfalfa-based diets compared to timothy-based diets. This indicates that additional amino acid catabolism occurred when horses consumed alfalfa in this study, which likely resulted in increased nitrogen losses. This provides evidence that additional amino acid intake from consuming alfalfa-based diets, compared to timothy-based diets, had no benefit on whole-body protein metabolism in these mature horses.

The next question became what effects would be seen in horses with elevated protein requirements fed primarily forage diets with a high or low protein ration balancer. Growing horses are accreting muscle and thus have greater amino acid requirements than

mature sedentary horses on a bodyweight-basis. They also have greater energetic needs on a bodyweight-basis, making it challenging to use a forage-only diet to support adequate growth. As a result, concentrate supplementation is a common strategy used in diet formulation for horses. Sedentary mature horses at maintenance are also commonly fed concentrates to ensure adequate intake of energy, vitamins and minerals. Improper balancing of diets can lead to horses consuming more crude protein than necessary in order to satisfy other nutrient requirements, and thus more amino acids are provided by the diet than required.

It was seen in the second study that growing horses had greater rates of phenylalanine use for protein synthesis, phenylalanine oxidation and phenylalanine flux when fed alfalfa-based diets, compared to consuming timothy-based diets. When growing horses received alfalfa-based treatments, more phenylalanine was consumed, when compared to timothy. This indicates that the amino acids consumed from alfalfa, compared to timothy, supported greater rates of protein synthesis in growing horses. Rates of phenylalanine flux were greater when yearlings consumed the high protein pellet compared to the low protein pellet. Phenylalanine oxidation and use for protein synthesis, however, were not different between protein levels of the ration balancer. This, combined with the plasma urea nitrogen concentrations increasing when horses consumed the high protein pellet compared to the low protein pellet, suggests that yearling horses derived no benefit from the high protein ration balancer. It was concluded that a diet based on alfalfa hay, compared to timothy hay, supplemented with a low protein ration balancer, compared to a high protein ration balancer, would support adequate protein synthesis while reducing dietary nitrogen losses in yearling horses.

One goal of this research was to determine if there were any benefits of a primarily legume versus a primarily grass forage-based diets on measures of whole-body protein metabolism. Another goal was to determine whether or not the addition of additional protein from a ration balancer or free amino acid supplementation would improve protein synthesis in growing and mature sedentary horses, respectively. In the current studies, if limiting amino acids were not met by the dietary treatments, then increased rates phenylalanine oxidation were seen. As limiting amino acid intake increased, then rates of phenylalanine oxidation decreased.

Mature horses in the first study were receiving 372% and 324% of their daily recommended lysine intake from the alfalfa treatment and lysine or + (Con) treatment and the timothy and lysine or + (Con) treatment, respectively (NRC, 2007). When horses received the (-) Con diet and alfalfa they consumed 270% of their daily recommended lysine intake and 222% when they received the (-) Con diet and timothy treatment (NRC, 2007). Lysine consumption exceeded recommendations in both forages when they were supplemented with glutamate, which could explain why no effects of supplemental lysine were seen. Growing horses in the second study were receiving 237% of the lysine requirement from the alfalfa and high protein treatment, 145% from the alfalfa and low protein treatment, 178% from the timothy and high protein treatment, and 86% from the timothy and low protein treatment (NRC, 2007). When growing horses consumed timothy and the low protein ration balancer, lysine intake was considered deficient based on the NRC's recommendation. This treatment did result in the lowest rate of phenylalanine use for protein synthesis and a lower than expect rate of average daily gain. However, although the average daily gain was numerically lower than the rates

supported by the other treatments, these differences were not statistically significant. Phenylalanine release from protein degradation was not measured, therefore definitive conclusions about whole-body phenylalanine balance could not be made. Whether horses consuming the timothy and low protein ration balancer were actually accreting less protein could not be determined either. However, the fact that there were no significant improvements in whole-body protein synthesis, even when the high protein ration balancer was fed, suggests that lysine may not have been limiting in this group.

Limiting amino acid intake does not equate to absorption, which is in part a product of the digestibility of the forages. A limitation of these studies is that the digestibilities of feeds provided were not obtained. More research is needed to define the limiting amino acids and their requirements in equine diets. This will allow for the optimization of diet formulation, with respect to protein, and ultimately reduce nitrogen losses to the environment. Reducing excess nitrogen excretion may positively benefit the environment, air quality and thus horse and human health, as well as mitigate fiscal losses. Understanding the mechanisms by which forage amino acids are digested and absorbed in the horse is another area warranting future research. Defining the bioavailability of essential and limiting amino acids provided by forages commonly fed to horses along the entirety of the gastrointestinal tract should also be investigated.

In conclusion, these studies demonstrated that a reduction in dietary nitrogen intake can maintain protein metabolism for maintenance in mature horses and growth in yearling horses. Because there were no benefits to whole-body protein synthesis with the addition of amino acid supplements, it can be concluded that forages and a small amount of concentrate provided enough of all the limiting amino acids to mature horses.

Although there were no apparent benefits between ration balancer protein levels, it is still unknown if a forage diet alone can provide enough of all the limiting amino acids to growing horses. When predominantly forage diets are used in growing horses, there appeared to be a benefit of feeding a forage higher in protein, such as alfalfa. Horses can be a challenging subject to research due to their large size and proportionately large nutritional needs. However, it is not difficult to provide all the nutritional needs of horses with increased requirements, such as yearlings, or in a maintenance state, such as sedentary mature horses, with primarily forage-based diets. Categorizing the amino acid digestibility of solely forage diets in these horses should be further researched as it provides a basis of digestibility of the largest component of the equine diet. The equine industry, the environment as well as horse and human health could benefit from research optimizing protein feeding with the goal of reducing nitrogen losses, but without compromising health or growth.

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Vita

Sophie A. Stratton

Education

University of Kentucky **August 2010-December 2015**

- Bachelors in Animal Science with an Equine Concentration *Lexington, KY*
- Dean's List *Fall 2014 and 2015*
- Undergraduate Research *Fall 2014*
  - Topic: Effect of Phenylbutazone on NF-κB production in the horse

University of Kentucky **August 2016-Current**

- Master of Science *Lexington, KY*
  - Animal & Food Sciences- Focus: Equine Nutrition
  - Thesis: Whole-Body Protein Metabolism in Mature and Growing Horses Receiving Predominantly Forage Diets
  - Expected Graduation: December 2018

Award

University of Kentucky **May 2017**

- 2nd Place *Lexington, KY*
  Annual Animal and Food Science's Graduate Association departmental poster symposium

Publication

Abstract Submitted **May 2017**

‣ C. M. M. Loos, T. Barnes, **S. Dorsch**, K.L Urschel. Effects of short-term dexamethasone treatment on glucose and insulin dynamics and muscle protein signaling pathways in horses, Journal of Equine Veterinary Science 52 (2017): 49-50; Equine Science Society Symposium, 2017, Minnesota

‣ **S. C. Dorsch,** T. Brewster-Barnes, A. M. Hannan, C. E. Harrell, C. M. M. Loos, and K. Urschel. Effects of alfalfa hay cubes versus timothy hay cubes on measured of whole-body protein metabolism in mature horses. *Journal of Equine Veterinary Science* 52 (2017): 77-78; Equine Science Society Symposium Proceedings, 2017, Minneapolis, Minnesota.

Manuscript in preparation:

‣ C.M.M.Loos, T.Barnes, **S. Dorsch**, K.L. Urschel. Effects of short-term dexamethasone administration on glucose and insulin dynamics and muscle protein signaling in horses after the consumption of a high protein meal.

‣ C.M.M.Loos, T.Barnes, **S. Dorsch**, K.L. Urschel. Effects of short-term dexamethasone administration on glucose and insulin dynamics and muscle protein signaling in horses after an oral sugar test.

‣ C.M.M.Loos, T.Barnes, **S. Dorsch**, A.A. Adams, K.L. Urschel. A high protein meal affects plasma insulin concentrations and amino acid metabolism in horses with equine metabolic syndrome.

‣ **S.A. Stratton**, A.M. Gerritsen, T. Brewster-Barnes, C.M.M.Loos, K.L. Urschel. Effect of forage type and amino acid supplementation on measures of whole-body protein metabolism in mature horses consuming a predominantly forage diet.

‣ **S.A. Stratton**, A.M. Gerritsen, T. Brewster-Barnes, C.M.M.Loos, K.L. Urschel. Effect of forage type and ration balancer protein content on measures of whole-body protein metabolism in growing horses consuming a predominantly forage diet.

Positions Held

Cheley Colorado Camps — **May 2016-August 2016**
Wrangler — *Estes Park, CO*

Veterinary Wellness Center — **December 2015-May 2016**
Receptionist/Vet Technician — *Lexington, KY*

Uptown Hounds — **August 2012-November 2015**
Receptionist/Pet Services Associate — *Lexington, KY*

University of Kentucky — **May-September 2014**
Undergraduate Research Assistant — *Lexington, KY*

Jump Start Farm — **May-September 2012**
Stable Hand — *Lexington, KY*

S.S. Eventing — **May-July 2011 & Winter 2012**
Stable Hand — *Georgetown, KY*

Internship

University of Kentucky — **June 2017-July 2017**
4H Intern for State Show — *Lexington, KY*